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HANDBOOK OF

CONTEMPORARY NEUROPHARMACOLOGY

Volume 1

Edited by

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PREFACE

Neuropharmacology is the study of drugs that affect the nervous system. This includes not only the identification of neuronal drug targets but also the study of basic mechanisms of neural function that may be amenable to pharmacological manipulation. Indeed, neuropharmacological drugs are commonly used as valuable tools to discover how nerve cells function and communicate in addition to therapeutic agents for the treatment of a wide variety of neuropsychiatric disorders. In fact, drugs that are used to treat disorders of the brain and nervous system represent one of the largest groups of approved therapeutic agents. Clearly the demand for drugs to treat disorders of the nervous system will only grow in the face of an aging population. Not surprisingly, almost all major pharmaceutical corporations and many biotechnology companies have extensive drug discovery programs in neuroscience and neuropharmacology. The recent pace of research and discovery in neuropharmacology and associated therapeutics has been quite rapid, as is true for most areas of biomedical research. Given this as well as the extremely broad nature of the field, we felt that it would be timely and important to develop a comprehensive handbook of neuropharmacology that would include state-of-art reviews covering both basic principles and novel approaches for clinical therapeutics.

Our approach for the organization of this handbook was primarily translational (bench to bedside) in nature. The three book volumes consist of 10 clinical sections, each consisting of 4–7 chapters devoted to various neuropsychiatric disorders, including mood, anxiety, and stress disorders, psychosis, pain, neurodegeneration, and many others. In most cases, these sections have introductory chapters providing background information and/or basic principles prior to presenting chapters covering state-of-the-art therapeutics. Volume I also contains a large introductory section consisting of 17 chapters on basic neuropharmacological subjects and principles. These include chapters on the history of neuropharmacology as well as intercellular and intracellular signaling followed by chapters covering all of the major neurotransmitter systems and other important signaling molecules, such as ion channels and transporters. Our objective for this project was to create a high-level reference work that will be useful to all practitioners of neuropharmacology ranging from graduate students, academicians, and clinicians to industrial scientists working in drug discovery. These volumes will be part of the John Wiley & Sons major reference work program and will be published online as well as in print. The online version of this handbook is expected to undergo frequent updates and additions in order to maintain its cutting-edge status.

The editors would like to thank all of the chapter contributors for their hard work and commitment to this project. We would also like to thank our managing editor, Jonathan Rose, at John Wiley & Sons for all of the valuable assistance that he has provided.

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Contents

Preface	xi
Contributors	xiii

VOLUME 1

PART I BASIC NEUROPHARMACOLOGY	1
Chapter 1 Soup or Sparks: The History of Drugs and Synapses	3
<i>William Van der Kloot</i>	
Chapter 2 Synaptic Transmission: Intercellular Signaling	39
<i>J. David Jentsch and Robert H. Roth</i>	
Chapter 3 Synaptic Transmission: Intracellular Signaling	59
<i>R. Benjamin Free, Lisa A. Hazelwood, Yoon Namkung Michele L. Rankin, Elizabeth B. Rex, and David R. Sibley</i>	
Chapter 4 Neuronal Nicotinic Receptors: One Hundred Years of Progress	107
<i>Kenneth J. Kellar and Yingxian Xiao</i>	
Chapter 5 Muscarinic Acetylcholine Receptors	147
<i>Jürgen Wess</i>	
Chapter 6 Norepinephrine/Epinephrine	193
<i>Megan E. Kozisek and David B. Bylund</i>	
Chapter 7 Dopaminergic Neurotransmission	221
<i>John A. Schetz and David R. Sibley</i>	
Chapter 8 Serotonin Systems	257
<i>John A. Gray and Bryan L. Roth</i>	
Chapter 9 Neuropharmacology of Histamine in Brain	299
<i>Raphaël Faucard and Jean-Charles Schwartz</i>	

Chapter 10	Ionotropic Glutamate Receptors	365
	<i>David Bleakman, Andrew Alt, David Lodge, Daniel T. Monaghan, David E. Jane, and Eric S. Nisenbaum</i>	
Chapter 11	Metabotropic Glutamate Receptors	421
	<i>James A. Monn, Michael P. Johnson, and Darryle D. Schoepp</i>	
Chapter 12	Pharmacology of the GABA_A Receptor	465
	<i>Dmytro Berezhnoy, Maria C. Gravielle, and David H. Farb</i>	
Chapter 13	Metabotropic GABA Receptors	569
	<i>Martin Gassmann and Bernhard Bettler</i>	
Chapter 14	Voltage-Gated Ion Channels	617
	<i>Alex Fay, Patrick C. G. Haddick, and Lily Yeh Jan</i>	
Chapter 15	Neuropeptides	669
	<i>Fleur L. Strand</i>	
Chapter 16	Neurotransmitter Transporters	705
	<i>Jia Hu, Katherine Leitzell, Dan Wang, and Michael W. Quick</i>	
Chapter 17	Gaseous Signaling: Nitric Oxide and Carbon Monoxide as Messenger Molecules	743
	<i>Kenny K. K. Chung, Valina L. Dawson, and Ted M. Dawson</i>	
PART II	MOOD DISORDERS	763
Chapter 18	Neurobiology and Treatment of Depression	765
	<i>Alexander Neumeister, Dennis S. Charney, Gerard Sanacora, and John H. Krystal</i>	
Chapter 19	Neurotrophic Factors in Etiology and Treatment of Mood Disorders	789
	<i>Ronald S. Duman</i>	
Chapter 20	Antidepressant Treatment and Hippocampal Neurogenesis: Monoamine and Stress Hypotheses of Depression Converge	821
	<i>Alex Dranovsky and René Hen</i>	
Chapter 21	Neuroendocrine Abnormalities in Women with Depression Linked to the Reproductive Cycle	843
	<i>Barbara L. Parry, Charles J. Meliska, L. Fernando Martinez, Eva L. Maurer, Ana M. Lopez, and Diane L. Sorenson</i>	
Chapter 22	Neurobiology and Pharmacotherapy of Bipolar Disorder	859
	<i>R. H. Belmaker, G. Agam, and R. H. Lenox</i>	
Index		877
Cumulative Index		915

VOLUME 2

PART I	ANXIETY AND STRESS DISORDERS	1
Chapter 1	Neurobiology of Anxiety	3
	<i>Miklos Toth and Bojana Zupan</i>	
Chapter 2	Pharmacotherapy of Anxiety	59
	<i>Jon R. Nash and David J. Nutt</i>	
Chapter 3	Benzodiazepines	93
	<i>Hartmut Lüddens and Esa R. Korpi</i>	
Chapter 4	Neuroactive Steroids in Anxiety and Stress	133
	<i>Deborah A. Finn and Robert H. Purdy</i>	
Chapter 5	Emerging Anxiolytics: Corticotropin-Releasing Factor Receptor Antagonists	177
	<i>Dimitri E. Grigoriadis and Samuel R. J. Hoare</i>	
Chapter 6	Neurobiology and Pharmacotherapy of Obsessive-Compulsive Disorder	215
	<i>Judith L. Rapoport and Gale Inoff-Germain</i>	
PART II	SCHIZOPHRENIA AND PSYCHOSIS	249
Chapter 7	Phenomenology and Clinical Science of Schizophrenia	251
	<i>Subroto Ghose and Carol Tamminga</i>	
Chapter 8	Dopamine and Glutamate Hypotheses of Schizophrenia	283
	<i>Bitá Moghaddam and Houman Homayoun</i>	
Chapter 9	Molecular Genetics of Schizophrenia	321
	<i>Liam Carroll, Michael C. O'Donovan, and Michael J. Owen</i>	
Chapter 10	Postmortem Brain Studies: Focus on Susceptibility Genes in Schizophrenia	343
	<i>Shiny V. Mathew, Shruti N. Mitkus, Barbara K. Lipska, Thomas M. Hyde, and Joel E. Kleinman</i>	
Chapter 11	Pharmacotherapy of Schizophrenia	369
	<i>Zafar Sharif, Seiya Miyamoto, and Jeffrey A. Lieberman</i>	
Chapter 12	Atypical Antipsychotic Drugs: Mechanism of Action	411
	<i>Herbert Y. Meltzer</i>	

PART III	SUBSTANCE ABUSE AND ADDICTIVE DISORDERS	449
Chapter 13	Introduction to Addictive Disorders: Implications for Pharmacotherapies	451
	<i>Mary Jeanne Kreek</i>	
Chapter 14	Dopaminergic and GABAergic Regulation of Alcohol-Motivated Behaviors: Novel Neuroanatomical Substrates	465
	<i>Harry L. June and William J. A. Eiler II</i>	
Chapter 15	Nicotine	535
	<i>August R. Buchhalter, Reginald V. Fant, and Jack E. Henningfield</i>	
Chapter 16	Psychostimulants	567
	<i>Leonard L. Howell and Heather L. Kimmel</i>	
Chapter 17	MDMA and Other “Club Drugs”	613
	<i>M. Isabel Colado, Esther O’Shea, and A. Richard Green</i>	
Chapter 18	Marijuana: Pharmacology and Interaction with the Endocannabinoid System	659
	<i>Jenny L. Wiley and Billy R. Martin</i>	
Chapter 19	Opiates and Addiction	691
	<i>Frank J. Vocci</i>	
PART IV	PAIN	707
Chapter 20	Neuronal Pathways for Pain Processing	709
	<i>Gavril W. Pasternak and Yahong Zhang</i>	
Chapter 21	Vanilloid Receptor Pathways	727
	<i>Makoto Tominaga</i>	
Chapter 22	Opioid Receptors	745
	<i>Gavril W. Pasternak</i>	
Chapter 23	Advent of A New Generation of Antimigraine Medications	757
	<i>Ana Recober and Andrew F. Russo</i>	
	Index	779
	Cumulative Index	817
VOLUME 3		
PART I	SLEEP AND AROUSAL	1
Chapter 1	Function and Pharmacology of Circadian Clocks	3
	<i>Gabriella B. Lundkvist and Gene D. Block</i>	

Chapter 2	Melatonin Receptors in Central Nervous System	37
	<i>Margarita L. Dubocovich</i>	
Chapter 3	Narcolepsy: Neuropharmacological Aspects	79
	<i>Seiji Nishino</i>	
Chapter 4	Hypocretin/Orexin System	125
	<i>J. Gregor Sutcliffe and Luis de Lecea</i>	
Chapter 5	Prokineticins: New Pair of Regulatory Peptides	163
	<i>Michelle Y. Cheng and Qun-Yong Zhou</i>	
Chapter 6	Sedatives and Hypnotics	177
	<i>Keith A. Wafford and Paul J. Whiting</i>	
PART II	DEVELOPMENT AND DEVELOPMENTAL DISORDERS	201
Chapter 7	Regulation of Adult Neurogenesis	203
	<i>Heather A. Cameron</i>	
Chapter 8	Neurotrophic Factors	221
	<i>Franz F. Hefti and Patricia A. Walicke</i>	
Chapter 9	Neurotrophins and Their Receptors	237
	<i>Mark Bothwell</i>	
Chapter 10	Tourette's Syndrome and Pharmacotherapy	263
	<i>Pieter Joost van Wattum and James F. Leckman</i>	
Chapter 11	Neuropharmacology of Attention-Deficit/Hyperactivity Disorder	291
	<i>Paul E. A. Glaser, F. Xavier Castellanos, and Daniel S. Margulies</i>	
Chapter 12	Psychopharmacology of Autism Spectrum Disorders	319
	<i>Adriana Di Martino, Steven G. Dickstein, Alessandro Zuddas, and F. Xavier Castellanos</i>	
PART III	NEURODEGENERATIVE AND SEIZURE DISORDERS	345
Chapter 13	Stroke: Mechanisms of Excitotoxicity and Approaches for Therapy	347
	<i>Michael J. O'Neill, David Lodge, and James McCulloch</i>	
Chapter 14	Epilepsy: Mechanisms of Drug Action and Clinical Treatment	403
	<i>William H. Theodore and Michael A. Rogawski</i>	
Chapter 15	Pharmacotherapy for Traumatic Brain Injury	443
	<i>Donald G. Stein and Stuart W. Hoffman</i>	
Chapter 16	Dementia and Pharmacotherapy: Memory Drugs	461
	<i>Jerry J. Buccafusco</i>	

Chapter 17	Pharmacotherapy and Treatment of Parkinson's Disease	479
	<i>Wing Lok Au and Donald B. Calne</i>	
Chapter 18	Parkinson's Disease: Genetics and Pathogenesis	523
	<i>Claudia M. Testa</i>	
Chapter 19	Invertebrates as Powerful Genetic Models for Human Neurodegenerative Diseases	567
	<i>Richard Nass and Charles D. Nichols</i>	
PART IV	NEUROIMMUNOLOGY	589
Chapter 20	Myelin Lipids and Proteins: Structure, Function, and Roles in Neurological Disorders	591
	<i>Richard H. Quarles</i>	
Chapter 21	Pharmacology of Inflammation	621
	<i>Carmen Espejo and Roland Martin</i>	
Chapter 22	Pharmacological Treatment of Multiple Sclerosis	671
	<i>B. Mark Keegan</i>	
Chapter 23	Novel Therapies for Multiple Sclerosis	683
	<i>Martin S. Weber and Scott S. Zamvil</i>	
Chapter 24	Neuropharmacology of HIV/AIDS	693
	<i>Sidney A. Houff and Eugene O. Major</i>	
PART V	EATING AND METABOLIC DISORDERS	731
Chapter 25	Leptin: A Metabolic Perspective	733
	<i>Dawn M. Penn, Cherie R. Rooks, and Ruth B. S. Harris</i>	
Chapter 26	Ghrelin: Structural and Functional Properties	765
	<i>Birgitte Holst, Kristoffer Egerod, and Thue W. Schwartz</i>	
Chapter 27	Mechanisms Controlling Adipose Tissue Metabolism by the Sympathetic Nervous System: Anatomical and Molecular Aspects	785
	<i>Sheila Collins, Renato H. Migliorini, and Timothy J. Bartness</i>	
Chapter 28	Antiobesity Pharmacotherapy: Current Treatment Options and Future Perspectives	815
	<i>Yuguang Shi</i>	
Index		845
Cumulative Index		881

1

SOUP OR SPARKS: THE HISTORY OF DRUGS AND SYNAPSES

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1.1	Action of Curare	4
1.2	Bernard's Career	5
1.3	Scientists, Patrons, and Money	7
1.4	A Changing World	7
1.5	German-Speaking Universities	9
1.6	Michael Forster	9
1.7	Gaskell and Langley	11
1.8	The Synapse	13
1.9	Dale and Loewi	14
1.10	Henry Wellcome	15
1.11	Ergot and Adrenaline	15
1.12	Histamine	16
1.13	Acetylcholine	17
1.14	National Institute for Medical Research	17
1.15	Loewi's Experiment	18
1.16	Feldberg	20
1.17	Nobel Prize of 1936	21
1.18	Electric Organ	22
1.19	Eccles, Kuffler, and Katz	22
1.20	Nachmansohn	23
1.21	Postwar Science	24
1.22	Bovet	25
1.23	Ion Channels	25
1.24	Soup, not Sparks	25
1.25	Actions of + TC	27
1.26	Synaptic Vesicles	27
1.27	Cloning The nAChR	27
1.28	How Can Chemical Transmission Be So Fast?	28
1.29	Eccles and Central Synapses	28
1.30	Adrenergic Transmitters in CNS	29
1.31	Carlsson	30

1.32	Second Messengers	31
1.33	Amino Acid Transmitters	32
1.34	Kuffler	32
1.35	End of the Era	33
1.36	Conclusions	34
	Acknowledgments	34
	References	35

1.1 ACTION OF CURARE

Claude Bernard (1813–1878; Fig. 1.1a) recalled that, “in 1845, Monsieur Pelouze gave me a toxic substance, called curare, which had been brought to him from America” [1]^a. He injected some into a frog. The frog became paralyzed. A “physiological dissection” showed that the heart was beating, blood was flowing, and the intestines were motile. He stimulated motor nerves with a shock from an inductorium powered by a battery made of alternating zinc and copper plates charged with vinegar. In the curarized frog the muscles contracted when stimulated, but not when the motor nerve was shocked. Curare might be blocking nerve conduction. To check this, he prepared a pithed frog, ligaturing off the arterial circulation to the legs but leaving their nerves intact. When he stimulated the nerve running down the leg, the muscles contracted. Then he injected curare. The upper part of the frog’s body became flaccid and respiration stopped. When he pinched the skin on the frog’s back, the legs were pulled up, just as in a normal frog. In the curarized anterior part of the frog the sensory nerves and spinal cord obviously worked even though the motor nerves did not. Muscles throughout the preparation contracted when stimulated directly. The drug acted on motor nerves. In isolated nerve–muscle preparations the muscle contracted when it or the nerve was stimulated. When the nerve was soaked in curare solution, the muscle still responded to nerve stimulation. When the muscle was soaked in the drug, it no longer contracted when the nerve was stimulated but did contract when stimulated directly. The poison does not block most of the motor nerve; it acts somewhere after the nerve has entered the muscle.

To us Bernard’s work with curare is straightforward science. To his contemporaries it was a dazzling display of how experiments can reveal how the body works and vivid proof that a drug may target a specific site in the body [3]. According to Bernard’s student, Paul Bert, Bernard had a “love of certainty” [4]. “He discovered as others breathed.” He was aware not only of “the endless multiplicity of unknown data in physiology, but also their subordination to the general laws of matter and their obedience to experimental method.” The thesis of this chapter is that working out precisely how curare acts provided the conceptual framework for much of neuropharmacology.

Bernard found that low doses of curare paralyzed the limb muscles but did not block the contractions of the diaphragm. These animals lived and recovered when

^aTo keep the bibliography within bounds many references are to reviews rather than original papers. Information about the lives and works of Nobel Laureates come from nobelprize.org. Information about individual drugs is from [2], which has excellent historical sections.

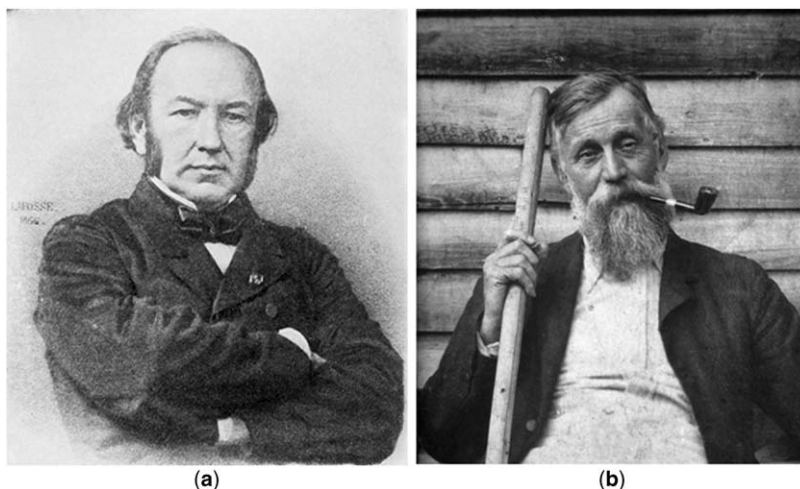


Figure 1.1 (a) Claude Bernard. (b) Sir William Foster; relaxing during a trip to the United States. (Courtesy of the Wellcome Library, London.)

they rid themselves of the drug. Curare introduced into the stomach did not poison the animal. When the gastric juice was removed and injected, it paralyzed the subject. Hence curare was not absorbed from or destroyed by the stomach. He considered curare to be “an instrument which dissociates and analyzes the most delicate phenomena of the living mechanism” [5].

His experiments were bedeviled by using different preparations of the drug; curare is a generic term for arrow poisons from the Amazon Basin [6]. After its discovery by Europeans it was years before Alexander von Humboldt (1769–1859) found an old native who had “the impassive air and pedantic tone formerly found in the European pharmacist” and who showed him how it was done. It was a water extract of the bark from a climbing vine of the genus *Strychnos*—which took a knowing eye to identify—with the water boiled off to leave a tar-like residue. By the 1880s chemists had found that there are different alkaloids in the curare from different regions which were categorized by their packaging: pot, calabash, and tube. The latter was the most potent and its active principle is (+)-tubocurarine chloride hydrate (+ TC). All but the finer points of its structure were worked out in the 1930s (Fig. 1.2).

1.2 BERNARD'S CAREER

Bernard presented his work on curare to the French Academy of Sciences, which met weekly. The academy was founded in 1666. In 1835 it began to issue the *Comptes Rendus*, where Bernard published [7]. He also wrote books describing his major discoveries, and his popular article on curare thrilled readers with his vivid description of the curarized animal: “All though the ages poetic fiction has sought to awaken our pity by representing living creatures locked in inanimate bodies” [8]. His early discoveries established his reputation and in 1854 he was appointed to a new chair in general physiology at the Sorbonne. It was a time when many universities were building up science. In 1858 he moved to the chair of medicine at the Collège de

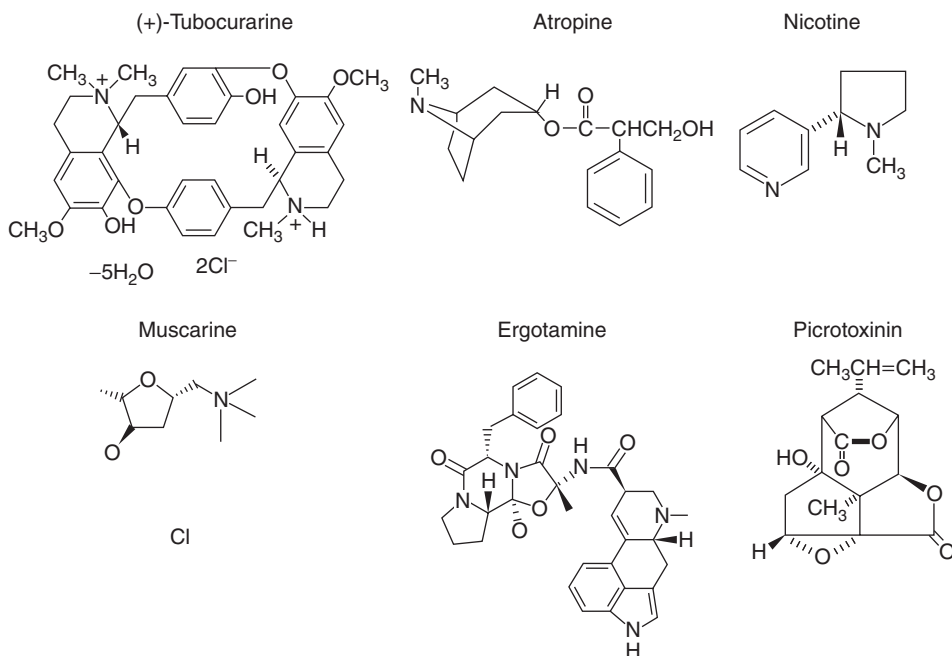


Figure 1.2 Some of the wealth of plant products that were starting points in the development of neuropharmacology.

France. The Emperor Napoleon III, after a lengthy talk at a social gathering, had a laboratory built for him in 1864. He joined the “40 immortals” in the Académie Française in 1868, and the next year was appointed to a seat in the Sénat.

He was born in the village of St. Julian to a family of Burgundian vintners [9]. His father was a poor school master. As a lad he wanted to be a writer. At 19 he came to Paris “with almost no paraphernalia except a tragedy which had never been acted and a farce-comedy which had some success at a small theater in Lyons” [4]. He carried an introduction to a prominent critic, who read his work and advised him to take up a profession and write on the side. In medical school he was a mediocre student; in the final examination he placed 26 out of 29. He had a talent for doing beautiful dissections, which is why the physiologist François Magendie (1783–1855) asked him to assist in preparing facial nerves for experiments. Magendie had demonstrated the separation of motor and sensory nerves in the roots of the spinal cord and in 1821 had founded the first physiological journal: the *Journal de Physiologie Expérimentale*. The leading French pharmacists were isolating potential drugs from plants; Magendie often tested their products. Had he tested curare it would probably have satisfied him to observe the paralysis. Once in a laboratory all of Bernard’s hitherto hidden talents flashed into view. He influenced many by his teaching. The scientific content of Bernard’s lectures was high, but organization might collapse. If his line of thought “escaped him, he followed it without rebellion, leaving his speech drooping, his lecture in confusion, while he listened softly to what it said to him” [4].

His personal life was deeply troubled. He married for money. His wife wanted him to practice medicine—as Magendie always did—so they would have a splendid

income. Bernard had time only for science. Even more troubling she was an ardent opponent of vivisection, writing her husband long letters denouncing his practices and enlisting their two daughters in her cause. When he fell ill at the end of 1877, he diagnosed his own doom [10]. Experiments, including some with curare, were unfinished: “What a pity, it would have been good to finish it” [4]. He was the first scientist given a state funeral in France.

The story of the action of curare I have presented so far is, as any working scientist will appreciate, far too simplified. There was an appreciable literature before Bernard, primarily showing the deadly effects of the poison were due to suffocation; artificial respiration kept curarized animals alive [6]. After Bernard published his results, Alfred Vulpian (1826–1887) proposed that the target for curare is the motor end plate of the muscle fibers, which had just been discovered [11, 12]. One of Bernard’s students then described in detail the motor end plates in lizard muscles and thought that he could distinguish those that had been exposed to strong curare solutions from normal [13].

1.3 SCIENTISTS, PATRONS, AND MONEY

There is a fundamental difference between the histories of science and scientists and those of art and artists or politics and politicians. Without Michelangelo the painting on the ceiling of the Sistine Chapel would be completely different; without Claude Bernard we would still know where curare acts—and all of his other discoveries as well. Scientists are placing pieces in a jigsaw puzzle that is predetermined by the laws of nature and evolutionary history—physical and organic. But this does not mean that great scientists are less important than great artists, musicians, or politicians. Bernard and his contemporary Louis Pasteur (1822–1895) led by sweeping the cobwebs of vitalism out of French biology. Their fame stimulated public interest in and support for science. Thinking of public support reminds us of another aspect of history that is too often slighted. Michelangelo and his assistants painted the ceiling of the Sistine Chapel, but only because of Pope Julius II. He compelled Michelangelo to move to Rome to repaint the ceiling and financed the project from the vast resources of the Catholic Church. Paying for the salaries, supplies, laboratories, and the like is even more important in science. Hence, as we go along, I will call attention to some patrons of science and to some of the sources of funds for paying for research.

1.4 A CHANGING WORLD

In Bernard’s lifetime Europe changed almost beyond comprehension. He came to a Paris of medieval streets. When he died it was crisscrossed by wide boulevards lined with stone-faced apartments and shops with large glass windows under wrought-iron balconies and shining at night in the light of gas lamps. Over the nineteenth century the population of Europe increased almost fourfold, despite millions emigrating for opportunities elsewhere. The world population had started growing rapidly at the beginning of the eighteenth century (Fig. 1.3a) [14]. A major reason for such rapid growth was improvements in agricultural practice. With better nutrition people lived longer. With more people who took in more energy for work there was an explosive

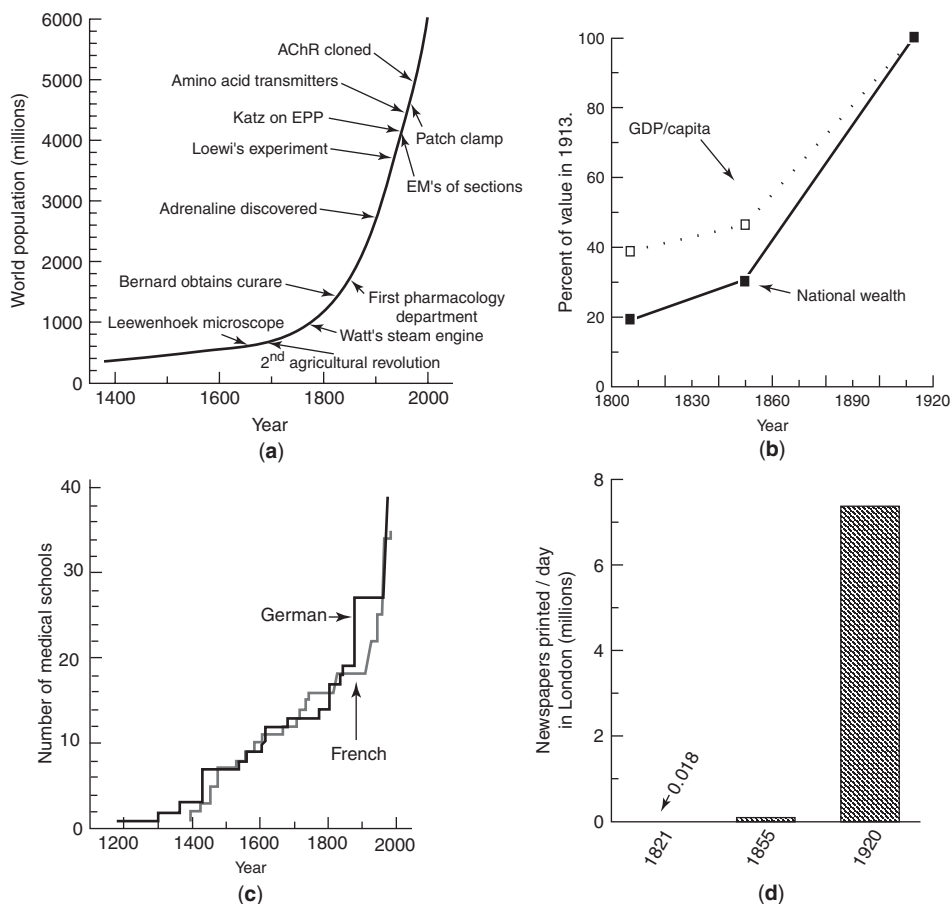


Figure 1.3 Some of the changes in the modern era. (a) World population. Note the change in rate at about 1700. To my mind the growth of science follows a similar curve. The estimated population at year 0 is about 200 million. (Data from Robert W. Fogel, Nobel Laureate in Economics, 1993) [14]. (b) The rise in the GDP in the Netherlands and the increase in national wealth. The Netherlands was chosen for the example because of the reliability of the data from the Groningen Growth and Development Centre [www.ggde.net]. (c) The numbers of still-existing medical schools in Germany and France as a function of time. (d) The number of newspapers printed on a weekday in London. The bar for 1821 is too small to be seen, so the figure is indicated above it [15].

expansion of industry. The energy of the workers was then supplemented by energy from fossil fuels trapped as steam. Over the nineteenth century the gross domestic product (GDP) per capita more than doubled (Fig. 1.3b). The increases in wealth and population led to the founding of universities and medical schools (Fig. 1.3c). The number of scientists was rapidly expanding. In France, the medieval universities had been torn asunder by the revolution. They were reformed and supplemented by the establishment of new schools specifically oriented to training in the professions. They were all supervised by the Université de Francé.

There was also an explosion in the availability of information. Steam presses slashed the cost of printing. There was an enormous increase in newspaper

circulation (Fig. 1.3d) [15]. Therefore many citizens knew of Bernard's work on curare and of advances in medical care, like the development of anesthesia—positive reinforcements for supporting science.

1.5 GERMAN-SPEAKING UNIVERSITIES

A political map of the Germany of 1800 shows a hodgepodge of kingdoms, principalities, bishoprics, and free cities united only by language and culture. During the Reformation the medieval universities had been taken over by the “states” [16, 17]. As population and wealth increased, so did support for the universities, and new ones were founded. Professors were expected to enlarge knowledge as well as to teach, and they decided what would be taught and what would be investigated. The goals for the universities had been enunciated by the first dean of the faculty of philosophy in Berlin, Johann Gottlieb Fichte (1762–1814), in a series of “Addresses to the German Nation” delivered in 1808–1809. Universities were built on three maxims: the freedom of the individual, of research, and of the nation. University life then was much like today; Fichte was the first rector in Berlin but soon had a conflict with the faculty and resigned.

The universities were open to all who had graduated from a classical high school, or “Gymnasium”. They had been started in 1815 and spread rapidly, and the States paid for education. There was no tuition, but some of the university lecture courses charged fees. The top 10% or so of the students worked for a doctorate, which supposedly attested to competence to work as a scholar in the field, though it was recognized that in medicine this ideal was seldom achieved.

A professorial chair was worth fighting for; as Max Weber (1864–1920) famously wrote, “many are called but few chosen”. There was a well-recognized pecking order among the universities, and professors competed to move up the ladder. The first pharmacology department was founded at the German-speaking University of Dorpat in Estonia in 1847 [18]. The first pharmacology department in Germany opened in 1849, and they spread rapidly throughout the country, partly because they were so useful to the booming German Chemical Cartel.

In 1870 Napoleon III declared war on Prussia. He found himself fighting all of the German states. After their victory they formed the German empire, and many of the states allocated part of their share of the enormous reparations extracted from the French to strengthen their universities. A major beneficiary was Strasbourg, in Alsace, which was taken over by Prussia. Oswald Schmiedeberg (1838–1921), who had studied at Dorpat, was appointed professor of pharmacology. He discovered that vagal stimulation slows the heart and became known as the “father of pharmacology” because 40 of his students obtained chairs. The professor of pathology was Bernhard Naunyn (1839–1925). In 1873 the two founded the *Archiv für Experimentelle Pathologie und Pharmakologie*.

1.6 MICHAEL FOSTER

Synaptic neuropharmacology would flourish in Great Britain—astonishing to anyone who had surveyed the situation there in 1850. Oxford and Cambridge were

assemblies of private corporate colleges, with their medieval, ecclesiastical roots largely unpruned [19]. Only members of the Church of England could be awarded degrees. The fellows were forbidden to marry. Pushed by German competition, they were transformed into scientific powerhouses by the leadership of men like Michael Foster (1836–1907; later Sir Michael; Fig 1.1b) [20]. Bernard was his inspiration; Foster wrote: “he has been to me as a father in our common science” [9].

Foster studied medicine at University College London (UCL)—known to its detractors as “the godless institution on Gower Street”—because he was a non-conformist. He spent a year in Paris but never “saw Bernard’s face.” After six years in medical practice he became an instructor at UCL and then Fullerian professor at the Royal Institution in London. He worked on the snail heart, trying unsuccessfully to determine whether beating is myogenic or neurogenic. The choice of preparation shows his affinity for Darwin’s ideas—evolution became bedrock in British physiology. He moved to Cambridge—without a university appointment—to a teaching position at Trinity College; this sidestepped the religious and marriage problems. Foster’s remit was to develop biological science. They gave him £400—roughly \$80,000 in the year 2000—for equipment, a room loaned by the university, and an assistant, Henry Newell Martin (1848–1893). A year after Foster’s arrival the religious tests at Cambridge and Oxford were abolished when Parliament, after years of acrimonious debate and reports by royal commissions, amended the charters to remove the religious requirement and to permit fellows to marry.

Foster invited interested students to see the body at work: muscles contracted, nerves conducted, exocrine glands secreted. Many found this thrilling and yearned to work on living things with their own hands. He persuaded the best to become botanists, histologists, embryologists, or physiologists—wherever there was a need. “He was a discoverer of men rather than facts” [21]. He sold research, “in this way only lies salvation”. He also provided scientists for other institutions. In 1876 he arranged for Martin to move to Johns Hopkins, which was just opening the first German-style university in the United States.

In 1878 Cambridge constructed a Biological School building, even though legally Foster and many of his staff were not part of the university. Finally in 1883, following a report by another royal commission and an act of Parliament, Foster was appointed professor of physiology. His *Textbook of Physiology*, first published in 1877, went through many editions in many languages, almost surely becoming the most widely used physiology textbook of the time [22]. It is filled with detailed descriptions of experiments and illustrations of the raw data. The 1893 edition concludes that it is not known how the motor nerve excites the muscle. Curare is not mentioned. There is no overview of the autonomic nervous system. Atropine, a product of the deadly nightshade, *Atropa belladonna*, blocks the action of the vagus on the heart and is said to act either on the muscle itself or on the “ultimate nerve endings” (Fig. 1.2). Atropine also blocks salivary and sweat gland secretions in response to nerve impulses and dilates the pupil. Muscarine is an alkaloid from the poisonous mushroom *Amanita muscaria*. Schmiedeberg was the first to isolate it and then he showed that it mimics the action of the vagus (Fig. 1.2). Drugs were known that acted on transmission between nerve and effector, but there was no integrating overview whatsoever.

In England the physiologists also taught histology. This was one of their strengths, since they all could do their own microanatomy. As his group grew, Foster turned the

teaching over to his protégés, a step that was welcomed by the students. Charles Scott Sherrington (later Sir Charles, 1857–1952, Nobel Laureate 1932), a student in the 1880s, found Foster “an appalling lecturer” and “never saw him do any demonstration” [21]. Often the young are not kind to their elders. Pharmacology came to Cambridge in 1899 in the form of Walter Ernest Dixon (1871–1931), who was appointed as an assistant to the Downing professor of medicine [23]. He had trained at St. Thomas’s Hospital London, where he worked on the addictive properties of cannabis and mescaline. “Pharmacological teaching in England, before Dixon began, was an unappetizing mixture of half obsolete *material medica* and empirical therapeutics. In Dixon’s hands it became a lively adventure in experimental science” [24]. The first pharmacology department in Britain opened in 1905.

Foster persuaded a rich student, Albert George Dew-Smith (1848–1903), to set up a company to manufacture apparatus. After a few years the firm was reorganized with Horace Darwin (1851–1928), Charles’s fifth son, as a partner and designer; the joint enterprise became the Cambridge Instrument Company [25]. There was a strong antivivisection movement in Britain, and in 1876 a law was enacted regulating the use of experimental animals. Concerned about how the law would operate, Foster organized the Physiological Society. It met periodically to discuss the workings of the law, but some scientific talk slipped in. Soon there were formal scientific sessions, which included the demonstrations Foster loved. Dew-Smith provided the financial support for starting the *Journal of Physiology*, which from the beginning had some foreign editors. Then Foster initiated international physiological congresses. The first was held in Basel in 1889. He served as secretary of the Royal Society from 1881 to 1903. His major achievement there was to persuade the fellows that it was appropriate for the society to provide scientific advice to the government.

1.7 GASKELL AND LANGLEY

Walter Holbrook Gaskell (1847–1914) followed his teacher Foster by working on the heart beat [26]. After years of careful work he showed that there are no nerve cells in the base of the ventricle of the turtle heart but that it could beat for at least 30 h when isolated—the beat is myogenic. Gaskell then worked on the structure of the involuntary nervous system, which he summarized in a book [27]. From there he turned to work on the evolutionary origin of the vertebrates. He inspired physiology students; “he spoke on controversial points with a half-suppressed enthusiasm which was eminently infectious” [24].

John Newport Langley’s (1852–1925; Fig. 1.4a) first paper began “Dr. Foster, having received from Dr. Ringer...a small quantity of the alcoholic extract of *jaborandi*, placed the drug in my hands and requested me to observe its physiological action” [28]. It was an extract of *Pilocarpus jaborandi*, a medicinal plant from South America, which contained the alkaloid pilocarpine. Langley found that pilocarpine slowed the frog heart or the beating of its isolated parts. Atropine antagonized pilocarpine. The plant was chewed to induce salivation. This led Langley to a 15-year study of salivary glands [29].

In 1890 he started working with the alkaloid nicotine, from the tobacco plant, *Nicotiana tabacum* (Fig. 1.2) [30]. When injected it produced a bewildering variety of

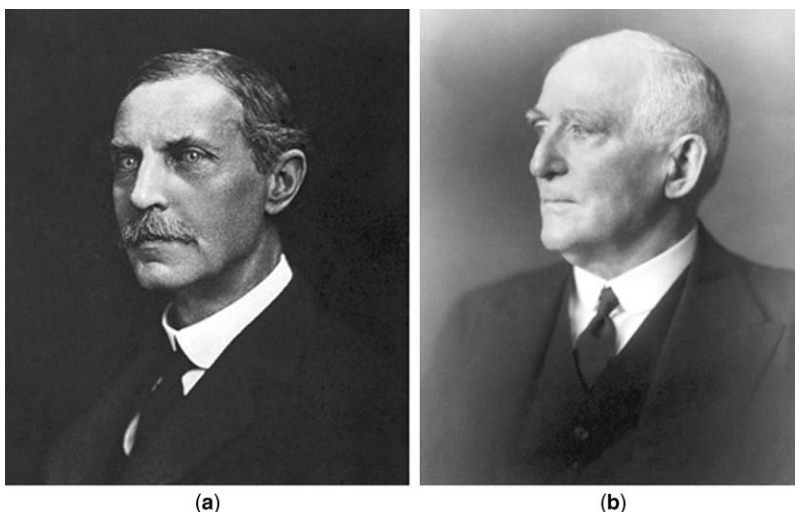


Figure 1.4 (a) John Newport Langley. (b) Sir Henry Wellcome. (Courtesy of the Wellcome Library, London.)

effects. He pinpointed sites of action by exposing a tissue, blotting it with a paper, and then—using a fine brush of sable hair—placing a $0.05\ \mu\text{L}$ drop of nicotine solution on its surface. He found that nicotine acted on autonomic ganglia, first exciting and then blocking, but had no effect on the pre- or postganglionic axons. Curare antagonized the stimulation by nicotine; atropine did not.

Working on frog and chicken skeletal muscles, he found that the results depended on the muscle chosen for study [31]. With a muscle like the frog sartorius, where there are seldom more than two end plates on a fiber, low concentrations of nicotine placed near the nerve endings elicited a brief burst of twitches. These are called twitch fibers. Following the contractions there was a block in neuromuscular transmission. The excitatory effect was blocked by curare at lower doses than required at the ganglia. Elsewhere on the twitch fibers there was no response unless the nicotine concentration was increased by orders of magnitude. Other muscles, like the frog rectus abdominis, are made up of fibers that are sensitive to nicotine almost all along their lengths, which became known as tonic fibers. Langley did not know that tonic fibers have end plates all along their length. The tonic fibers give a sustained contraction when nicotine is applied. Other muscles contain a mixture of the two types of fibers, so their response to nicotine was bewildering indeed. It did not faze Langley, who preached “get facts, then theory will take care of itself” [20]. Muscle denervated 100 days earlier still responded to nicotine. His view on the mechanism was based on Paul Ehrlich’s (1854–1915; Nobel Laureate 1908) hypothesis that protoplasm is one giant molecule and that drugs act on its side chains. Hence Langley concluded that the drug acts “directly on the muscle. In the muscle there are two substances to take into account, the sarcoplasm and the differentiated contractile substance. ...I take it that the contractile molecule has a number of ‘receptive’ or side-chain radicals, and that nicotine, by combining with one of these causes tonic contraction, and by combining with another, causes twitching, and that the latter is a much less stable part of the molecule” [31].

He summarized the anatomy and physiology of the autonomic nervous system in a book in which he described the evidence for two components, which he named sympathetic and parasympathetic [32]. The effects of parasympathetic stimulation are mimicked by muscarine, pilocarpine, and choline and blocked by atropine. He called these cholinophil. Most effects of sympathetic stimulation are mimicked by adrenaline (see below) and were called adrenophil. Sympathetic stimulation causes sweat glands to secrete but adrenaline does not. Sweating is blocked by atropine and stimulated by choline and the rest. In his view, there were cholinophil and adrenophil receptors, but they could be either excitatory or inhibitory depending on the chemical changes in the tissue when the drug combined with the receptor.

Langley edited the *Journal of Physiology* for 30 years. Edit is too light a word. Almost invariably he would heavily correct papers and sometimes he would rewrite them completely. When Foster left, Langley became professor of physiology. He had much to do. The staff was excellent, the facilities deplorable. Three investigators shared a former coal cellar. Finally in 1914 a splendid new building was opened paid for by a London company, the Drapers'. Companies such as this are descendents of the medieval guilds which are now given over to good fellowship and good works. His research was supported by grants from the Royal Society and the British Medical Association. He inspired students by how relentlessly he worked at a problem and with his success in putting together the puzzle, not by warmth of personality. Langley was a brilliant figure-skater. One student thought this caught his essence: "brilliant technique on an icy background" [29].

1.8 THE SYNAPSE

Langley's papers at the beginning of the twentieth century vividly show the uncertainties about the pathway from nerves to muscles or glands. Was there cytoplasmic continuity? Were cells surrounded by membranes, and if so was there a gap between the nerve and the structure it innervated? The best microscopes revealed no membrane and no gap. Most electrophysiologists were convinced that the unseen membranes existed and thought that nerve impulses were transmitted by momentary changes in their properties [33]. In 1897 Sherrington proposed that the attributes of reflexes are due to the properties of the junctions between individual nerve cells, at structures he called synapses [34]. He pictured the synapse as a gap between the neurons bounded by the apposing membranes of the two cells. Synapses accounted for one-way conduction in the reflex arc and for the delay added onto the passage of the nerve impulses traversing the arc. However, synaptic delay is extremely short and transmission can be repeated at short intervals. Too fast, they argued, for a chemical reaction between a hypothetical transmitter and receptor, let alone further chemical reactions to produce an effect. Transmission must be electrical, just as it is down the nerve axon.

Camillo Golgi (1843–1926) and Santiago Ramón y Cajal (1852–1934) shared the Nobel Prize for Physiology or Medicine in 1906 for their work on the structure of neurons. They developed treatments that stained randomly selected neurons in their entirety: cell bodies, axons, and dendrites. Cajal argued that therefore there is no continuity between the cytoplasm of the individual neurons. As he said in his lecture, "it must be admitted that the nerve currents are transmitted from one

element to another as a consequence of a sort of induction or influence from a distance.” Golgi was more cautious, suggesting that at least some parts of the brain were made of an extensive reticulum rather than individual cells.

The Nobel Prizes, first awarded in 1901, rewarded outstanding investigators and publicized scientific progress. Citizens were elated when a fellow countryman won a Nobel Prize and were willing to see more of their taxes go toward science so they might do better in the international competition.

Ross Harrison (1870–1959) did not receive the Nobel Prize voted for him because none were awarded in 1917 due to the “Great War” [35]. Harrison placed bits of frog spinal cord in a drop of lymph on a sterile cover slip sealed over a hollow slide. For weeks he watched axons grow out from a ganglion cell, sometimes at a rate of 50 $\mu\text{m}/\text{h}$. The axons grew out to muscle fibers in the culture, which then contracted spontaneously [36]. There must be a synapse between the motor nerve and muscle.

1.9 DALE AND LOEWI

Among the Cambridge students inspired by Langley and Gaskell was Henry Hallett Dale (1875–1968; later Sir Henry) [37]. He did well in physiology and was awarded a research fellowship which enabled him to spend two years preparing a thesis as a candidate for a fellowship at Trinity College. Under Langley’s direction Dale did a morphological census of the various classes of fibers in a mixed nerve. It was dull work and did not get him the fellowship. Disappointed, he moved to London for the clinical part of the medical course. The clinicians “felt it obligatory to speak with a kind of oracular authority” with little scientific basis [37]. He won a fellowship to work in the Physiology Department at UCL. The fellowship was endowed by the writer George Eliot (1819–1880) in memory of her long-time companion George Henry Lewes (1817–1878). The professor was Ernst Henry Starling (1866–1927), who was then collaborating with his brother-in-law William Maddock Bayliss (1860–1924; later Sir William). They had just discovered the first hormone, secretin. Dale was asked to study the effects of large doses of secretin on the histology of the pancreas, work which he did not enjoy.

In 1902 Otto Loewi (1873–1961) came over from Germany for a few months to meet British scientists and to improve his English [38]. Dale tried to help with words and phrasing, but soon learned that his enthusiastic friend was not interested: “No. I have no time to learn English correctly; I wish to speak it fast.” The British were charmed with his fractured outpourings: “some of his achievements in that line have had a legendary survival, still being known... 60 years later.” Loewi also spent several weeks with Langley in Cambridge. At that time Loewi was an assistant in the Department of Pharmacology at Magdeburg, where the professor was Hans Horst Meyer (1853–1939). Meyer had shown that the deadly tetanus toxin is taken up in nerve endings and transported up the axons into the central nervous system (CNS), where it acts. Then he turned to kidney pharmacology. Loewi liked working with drugs, which he defined as “chemicals which when injected into an animal or applied to a tissue result in the publication of a scientific paper.”

Loewi had attended the University of Strasbourg where, at the insistence of his parents, he enrolled in medicine. Initially he “played hooky” from medicine, favoring lectures on architecture and philosophy which he loved [39]. One day, to obtain a

required signature, he went to Naunyn's class, hoping to get the signature before the lecture began. He was late, so he stood in the doorway waiting for it to be done. He was fascinated by the lecture, so he came again and became an enthusiastic student of medical science. Schmiedeberg directed his doctoral thesis. Loewi was surprised by the crowding and poor equipment in the British laboratories. In the German universities there was no fretting about funds or writing of grant proposals. The governments paid the bills but gave them "far-reaching autonomy," accepting "the universities proposals and paying the endowments and debts of the departments. ...If the debts were excessive the department got a warning—and a little later the payment" [39].

After Loewi returned home, Dale visited Germany: first to Magdeburg and then to Frankfurt-am-Main, where he spent four months working in Ehrlich's department. There "I had no results of my own worthy to record" [37]. Back in London, he had a job at UCL, at £150 per year, less than his fellowship had paid, so he was receptive when approached by Henry S. Wellcome (1853–1936; later Sir Henry; Fig. 1.4b), the proprietor of the huge pharmaceutical company Burroughs Wellcome.

1.10 HENRY WELLCOME

Wellcome had grown up in a farming community in Minnesota, where as a lad he worked in his uncle's drug store [40]. The office above was occupied by William James Mayo (1861–1939), who with his brother later established the Mayo Clinic. He encouraged young Wellcome to attend the Philadelphia College of Pharmacy, considered the best in the country. There he met Silas Burroughs (1846–1895). After graduation Wellcome worked in Peru and Ecuador, obtaining specimens of plants the inhabitants used medicinally. Burroughs had moved to London. He invited Wellcome to join him there in a company which would manufacture the new compressed medicinal pills. The compression process had been developed by an English artist for making better lead pencils. In Britain most physicians were still rolling their own pills. The Burroughs Wellcome Company made the pills and advertised them vigorously in professional journals, and by the time Wellcome was 30 the company had facilities around the world and he was very rich. The partners fought bitterly and were dissolving their partnership when Burroughs died of pneumonia. Wellcome became sole proprietor.

Wellcome understood that only research could expand the pitifully short list of useful drugs, so he set up The Wellcome Research Laboratories in Physiology in a southern suburb of London. They started by producing diphtheria antitoxin in horses. The antitoxin had been developed at the Pasteur Institute, but Wellcome was the first quantity producer. For its first 10 years the laboratory did no research.

1.11 ERGOT AND ADRENALINE

Wellcome wanted some real research done at his laboratory so he offered Dale £400 per year (equivalent to about \$60,000 in 2000). Dale's advisers all warned him that "I would be selling my scientific birthright for a mess of commercial potage" [24]. Still he would have "a marrying income" and be free from teaching, so he accepted, with the

proviso that his salary would be raised to £600 in two annual increments. Wellcome suggested that “when convenient, he would be glad to have me make some investigations on the pharmacology of ergot” [37]. Parke Davis & Co advertised that its ergot preparation was biologically standardized and Wellcome was eager to compete. Ergotamine is produced by a fungus, *Claviceps purpurea*, which infests rye and other grains (Fig. 1.2). It causes gangrene of the extremities, along with agonizing burning pain. Its toxicity had been known to the Assyrians, and it had been used by midwives to bring on delivery when labor was delayed well before it was adopted by physicians.

Wellcome was also interested in marketing adrenaline, which had been discovered in 1895 when Oliver and Schäfer injected an adrenal extract and recorded an increase in blood pressure that almost shot the mercury out of their manometer. Dale knew a great deal about adrenaline, because a close friend from Cambridge, Thomas Renton Elliott (1877–1961), had worked on it [41]. Following earlier work by Langley and others, Elliott showed: “In all vertebrates the reaction of any plain [smooth] muscle to adrenalin is of a similar character to that following excitation of the sympathetic (thoracico-lumbar) visceral nerves supplying that muscle. The change may be either contraction or relaxation” [42]. Denervated smooth muscles that had been innervated by the sympathetic system still respond to adrenaline and their sensitivity is enhanced. The last sentence of Elliott’s abstract when he presented this work to the Physiological Society was: “Adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery” [43]. This audacious idea did not make it into his paper, probably because Langley did not like it. When Elliott finished his work with adrenaline, he did not receive a fellowship so he entered medical school in London.

The link between ergot and adrenaline was discovered by Dale. One day he was measuring the effects of graded doses of ergot extract on the blood pressure of a spinal cat. When the tests were almost finished a sample of dried adrenals from the Burroughs Wellcome factory was delivered. He was to determine its adrenaline content. No need to waste the cat. When Dale injected the adrenal extract, the blood pressure fell, “and with the confidence of inexperience I condemned the sample without hesitation.” The same result a week later made him realize that he had made a “really shocking howler.” Ergot blocked excitatory effects of adrenaline without touching its inhibitory actions. It also blocked excitatory effects of sympathetic nerve stimulation. It did not block inhibitory effects of adrenaline or of nerve stimulation. Shortly thereafter, Dale was named the director of the laboratory.

He and George Barger (1878–1939), a chemist at the Institute, then studied more than 50 amines for what they christened their “sympathomimetic” actions—another term that has become part of the language. The closer the resemblance to adrenaline, the more potent the amine. Noradrenaline is a stronger exciter and weaker inhibitor than adrenaline. At the time noradrenaline was not known to occur in the body, so they passed over the implications.

1.12 HISTAMINE

One of the amines they worked with was histamine, which they had found in their ergot extracts and later showed was produced by bacterial action on histidine. It stimulates many smooth muscles but when injected causes a dramatic fall in blood

pressure: Its effects are much like anaphylactic shock. A few years later colleagues at the Institute showed that histamine is in the body. Working on the isolated guinea pig uterus one day, comparing the stimulation of contraction caused by histamine and by horse serum, Dale was startled to find that the serum was working at unexpectedly low concentrations. He inquired about the previous use of the guinea pig and learned that it had been injected with small amounts of horse serum in an assay of antitoxins. He picked up the clue and showed that the previous exposure to the serum *in vivo* sensitized the smooth muscle to subsequent challenges with serum: the basis for anaphylactic shock.

1.13 ACETYLCHOLINE

Working one day with a crude extract made from ergot on the spinal cat, Dale was startled to see the blood pressure fall to zero. He thought he must have clumsily injected enough air to stop the heart pumping: “And then, as I was hanging up my blouse, assuming that my mornings work was done, I caught sight of the recording drum out of the corner of my eye, and saw that the manometer float was rapidly returning to the level of the blood pressure record before the injection” [43a]. A second injection also temporarily stopped the heart. He injected atropine. Now the extract had no effect. He told his chemistry colleague, Arthur James Ewins (1882–1957), that the extract contained a substance that acted like muscarine. Ewins promptly isolated the base as a platinum salt, but determining its structure with a tiny sample was a formidable task. “That evening, in fact as I was getting into bed,” Dale remembered a report he had read eight years before that low concentrations of acetylcholine (ACh) slowed the heart. The next morning he asked Ewins “to get to work and make me some acetylcholine as soon as possible... Before many hours had passed we knew beyond doubt that the active principle we had found in the ergot salt was, indeed, physiologically indistinguishable from acetylcholine” [43a]. With this hint, Ewins identified his platinum salt as that of ACh.

Soon they showed that ACh acted like muscarine on heart, smooth muscle, and glands. And both were antagonized by atropine. Like nicotine it stimulated secretion from the adrenal medulla, where the ACh action was blocked by paralyzing doses of nicotine. They coined the terms *nicotinic* and *muscarinic* to describe the two actions of ACh. They were tantalizingly close to the answer: the result “gives plenty of scope for speculation.” But they did not think of an experiment to make the leap.

1.14 NATIONAL INSTITUTE FOR MEDICAL RESEARCH

Meanwhile Dale had moved. In 1908 the Liberal government in Britain passed a budget in the Commons that initiated unprecedented social reforms, a vast program modeled on what the Germans had achieved in Bismarck’s time. They established old-age pensions, workman’s compensation, an 8-h workday for miners, medical inspection of children, and unemployment insurance. The budget was only implemented after a prolonged fight with the House of Lords and two general elections. In 1912 the emboldened liberals included national health insurance in the budget. A small fraction of the premiums— “one penny per head per annum per insured

person”—was set aside for medical research. The government established the Medical Research Council (MRC) to spend the funds.

Dale was appointed director of the Department of Biochemistry and Pharmacology at the National Institute for Medical Research (NIMR) in London, which was established by the MRC. Dale was delighted to leave Wellcome, who too often made promises that were not implemented because he headed off on an expedition—he was an ardent archeologist and collector—before leaving instructions. The development of the NIMR was sidelined by the outbreak of World War I. Dale worked on antiseptics, antiamebic dysentery agents, and “wound shock,” concluding that treatment should be by “the addition by transfusion to the circulating blood of a fluid...—blood or plasma” [43a]. In 1920 the NMIR finally moved into quarters in Hampstead. Dale and his family were already living in a small manor house on the site rented from the MRC. Dale became its director in 1928.

1.15 LOEWI'S EXPERIMENT

The decisive experiments on synaptic transmitters were started on the Monday after Easter in 1920 at 3:30 in the Pharmacological Laboratory of the University of Graz Austria; Loewi had become professor there in 1909. Here is the experiment in his words [39]. (Note that a Straub canula is a glass tube that can be slipped though the stump of the aorta into the single ventricle of the frog's heart.) “The hearts of two frogs were isolated, the first with its nerves, the second without. Both hearts were attached to a Straub canula filled with a little Ringer solution. The vagus nerve of the first heart was stimulated for a few minutes. Then the Ringer solution that had been in the first heart was transferred to the second heart [Fig. 1.5a]. It slowed and its beats diminished just as if the vagus had been stimulated. Similarly when the accelerator nerve was stimulated and the Ringer from this period transferred, the second heart speeded up and its beats increased.” How clever to use frog hearts both for releasing the chemicals and performing the bioassays.

He called the substance released by vagal stimulation *vagusstoff*. It disappears rapidly if left in the ventricle and its effect on the second heart is blocked by atropine. They tested pilocarpine, muscarine, choline, and ACh by placing solutions in a heart for a time and then seeing whether the solutions had lost their potency when transferred to a second heart. Only ACh did. They then showed that hearts contained an esterase that broke down ACh. The esterase was inhibited by low concentrations of physostigmine, an alkaloid from the seeds of *Physostigma venenosum* from West Africa. It had been isolated in 1864 and used in the treatment of glaucoma since 1877. Loewi and his group showed that physostigmine inhibits what became known as acetylcholinesterase (AChE). Hence, when the effects of nerve stimulation on an organ were enhanced by physostigmine, it was likely that ACh was being released.

Years passed before Loewi's experiment was generally accepted as reproducible. It is not as easy as it sounds. Loewi was lucky because the winter frogs he started with had low levels of AChE; frogs are seasonal animals. All of the experiments he reported were done in February and March [44]. Usually AChE must be inhibited for the experiment to work, so there were many failures around the world. He submitted

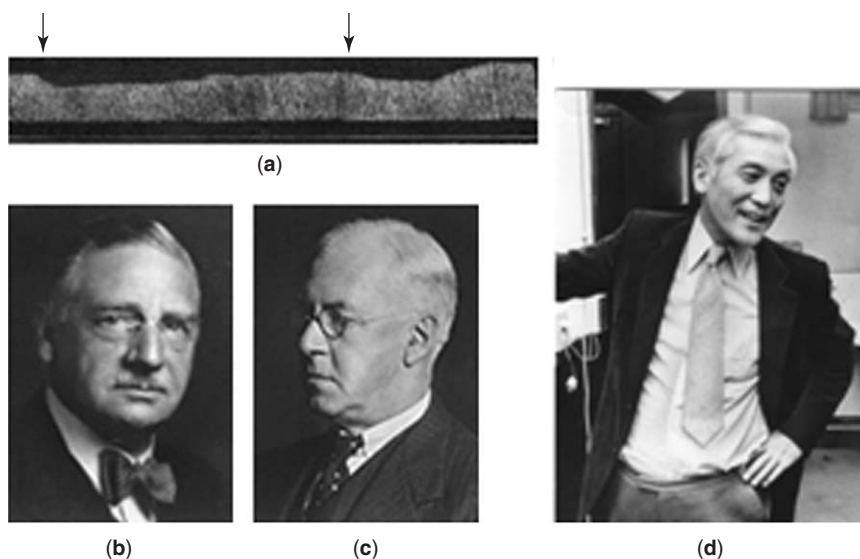


Figure 1.5 Great discoverers. (a) The first published record of the Loewi frog heart experiment. At the arrows the fluid in the heart was replaced with Ringer withdrawn from a heart whose vagus had been stimulated for 15 min. (b) Otto Loewi. (c) Henry Dale. ([b,c] copyright © The Nobel Foundation.] (d) Shosaku Numa.

his paper on 20 March, 1921, almost a year after the initial experiment, presenting results from 14 experiments on frogs and 4 on toads. There must have been many failures during the summer. Finally in 1926 he was invited to supervise a demonstration at the International Congress of Physiology in Stockholm. Happily—unlike so many demonstrations—it worked 16 times on the same hearts. He was required to stand away from the apparatus, because some claimed that he dispensed ACh from under his fingernails.

The experiment of stimulating the sympathetic supply to the heart and transferring the excitation in the Ringer solution was easier to reproduce, because the released transmitter is not dealt with rapidly. The effect of the excitatory chemical was blocked by ergotamine, which also antagonizes adrenaline. The potency of the transferred Ringer was increased by doses of cocaine that by themselves were ineffectual; cocaine was known to potentiate adrenaline. The material in the Ringer had the characteristics of adrenaline. Exceptionally frog sympathetic postganglionic nerve fibers release adrenaline rather than noradrenaline.

A question Loewi delighted in chewing over was how he had the idea for his experiment in the first place, as he described it: “The night before Easter Sunday... I awoke, turned on the light, and jotted down a few notes on a tiny slip of thin paper. I fell asleep again. It occurred to me at six o'clock in the morning that during the night I had written something most important, but I was unable to decipher the scrawl. The next night, at three o'clock, the idea returned. I got up and immediately went to the laboratory.” [39]. It may have grated on his competitors that this was his first major venture into neuropharmacology. His previous work had been on nutrition, kidney pharmacology, and the effects of digitalis on the heart.

1.16 FELDBERG

Adolph Hitler (1889–1945) became the chancellor of Germany early in 1933. The Achilles' heel in the German university system was that professors were civil servants. Every Jew or person of Jewish ancestry teaching in a German university was fired. Wilhelm Sigmund Feldberg (1900–1993) was told by the professor of pharmacology in Berlin to leave immediately and never to come back [45]. Hundreds of others had similar dismissals. A few weeks later Feldberg met with a representative of the Carnegie Foundation who had a list of opportunities abroad for displaced German scholars. Regretfully, none fit Feldberg. Feldberg mentioned that if an opening surfaced he felt sure Sir Henry Dale would recommend him. This jogged the representative's memory. Searching his notes he saw that that Dale had told him that there was a place for Feldberg at the NIMR [46].

Feldberg had spent two years in England, first with Langley and then with Dale. Now he returned with a wonderful present: an ultra sensitive bioassay for ACh measuring the contractions of the physostigmine-treated dorsal body wall muscle of the leech. It is so sensitive that they could take the venous effluent from a mammalian tissue in a physostigmine-treated animal, cool it, and let it flow over the leech muscle, which contracted if ACh had been released. Feldberg and co-workers in Berlin had just shown that ACh is released in the adrenal medulla when the sympathetic preganglionic nerve is stimulated.

Dale had seldom come to the laboratory after he had become director—now he was there every free moment. He had assembled a formidable group of co-workers. John Henry Gaddum (1900–1965) had turned from medicine to pharmacology when a girl at a ball told him of an opening in Dale's laboratory [46, 47]. George Lindor Brown (1903–1971) was an Oxford-trained electrophysiologist recruited to expand the available techniques [48]. He had finished constructing his electronics and was ready to go. Soon they were joined by Marthe Louise Vogt (1903–2002), a skilled microdissector who left Germany voluntarily because she so detested the Nazis.

There was a stream of discoveries. The cat superior cervical ganglion released ACh when the preganglionic nerve was stimulated. The amount that could be released was larger than the initial ACh content, so ACh is synthesized in the ganglion. So much ACh was released that the reinjection of outflow stimulated the postganglionic fibers—therefore the ACh released surely stimulated them also. Some postganglionic sympathetic fibers, like those innervating sweat glands, release ACh. Dale coined the terms *adrenergic* and *cholinergic*.

Stimulation of the motor axons liberated ACh at skeletal muscles. When the muscle was denervated, vigorous contraction elicited by direct stimulation did not release ACh. ACh was still released when contraction was blocked with +TC. Frustratingly they could not get a muscle to contract when ACh was injected into its arterial circulation. Reasoning that the ACh reaching the tiny end-plate region would be dilute: "We attempted a nearer approach to these supposed conditions of its natural release, by a method which enabled us, after a brief interruption of the arterial blood supply, to inject a small dose of acetylcholine, in a small volume of saline solution, directly and rapidly into the empty blood vessels of the muscle. The responses which we thus obtained were of an entirely different kind from any which had previously been recorded. A dose of about 2 gamma of acetylcholine, thus injected at close range into the vessels of a cat's gastrocnemius, produced a

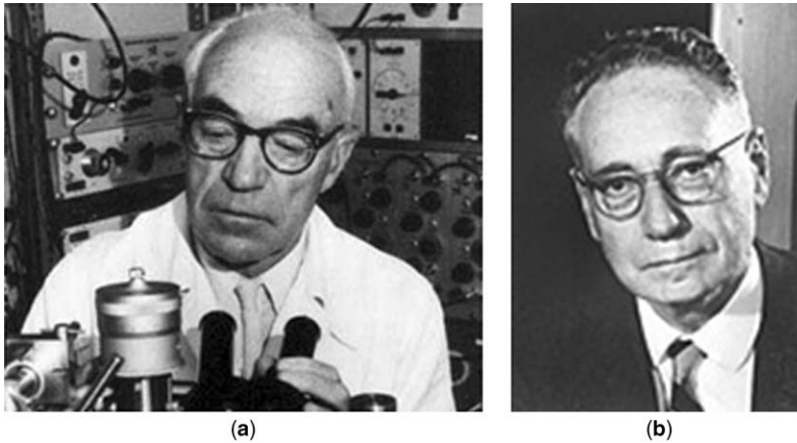


Figure 1.6 (a) Sir John Eccles. (b) Sir Bernard Katz (copyright © The Nobel Foundation.)

contraction with a maximal tension equal to that of the twitch produced by a maximal motor nerve volley, and of a rapidity but little less than that of the motor nerve twitch.” Vogt did the difficult dissections. Brown showed that the close injection elicited a short burst of action potentials in the muscle fibers. ACh was still released when contraction was blocked with +TC. Twenty-four papers and abstracts on ACh from Dale’s laboratories were published from 1934 to 1936. Feldberg was an author on all of them, because he did the assays.

Their results were presented at meetings of the Physiological Society. Not everybody liked them. The criticism was led by John Carew Eccles (1903–1997; later Sir John; Fig. 1.6a), a dynamic Australian who was always primed for the attack [49]. After graduating in Medicine from Melbourne, he had come to Oxford as a Rhodes Scholar in 1925. His sport—one of the selection criteria for a Rhodes—was pole-vaulting. He started graduate study in 1927 under Sherrington. Speed still made chemical transmission at fast synapses seem impossible—it was “soup” versus “sparks.”

1.17 NOBEL PRIZE OF 1936

The Nobel Prize for Physiology or Medicine in 1936 went to Loewi (Fig. 1.5b) and Dale (Fig. 1.5c) “for their discoveries relating to chemical transmission of nerve impulses.” In his lecture in Stockholm Dale cited 11 papers from the group; he was an author on 5. Naturally enough some searched the literature to cite those who had scooped the Laureates. Chemical transmission had been suggested repeatedly, starting with Emil Heinrich Du Bois-Reymond (1818–1896), who had been a professor in Berlin. Such diggings are a diversion. It was not hard to think that transmission might be chemical; it was hard to devise experiments to test the hypothesis.

When the Germans entered Austria in 1938 Loewi and two of his sons were jailed until he gave up his Nobel Prize money as ransom. After several years in temporary positions he became a research professor at New York University (NYU). His wife was kept in Austria until 1941, when she turned over her family property in

Italy to the Nazis. The Nazis devastated the German universities and enhanced those abroad. In Britain it seemed to one younger neuroscientist “that through most of my scientific lifetime the most distinguished of my seniors mostly spoke with guttural accents” [50].

1.18 ELECTRIC ORGAN

The electric organ of *Torpedo* is made up of a huge stack of motor nerve endings and end plates in series. Albert Fessard (1916–2003) worked with the preparation, so Feldberg traveled over to France to collaborate [51]. They set up the spinal preparation in a shielded cage. The amplifiers, recording apparatus, and engineer were outside. Only Feldberg was within, cautioned not to touch the animal—doing so would generate a nasty electrical artifact. He was to inject ACh in the artery and call out “now.” He called but the engineer hastily stopped the recording. A huge artifact. The engineer was worried about damaging his amplifier. Feldberg was put into rubber gloves and boots as insulation. “Now.” Another artifact. Feldberg asked for another try. “Now.” No response. “Fine,” said Feldberg, “I injected saline.” With the next “now” he injected ACh; there was the already familiar huge voltage spike, which they now knew was the discharge of the electric organ [46].

The biochemist David Nachmansohn (1899–1983) was in France working on the electric organ [52]. He had trained with Otto Meyerhoff (1889–1951; Nobel Prize 1922) in Berlin but had been driven out by the Nazis. The electric organ had been found to contain astonishing concentrations of AChE. Nachmansohn began to purify it.

1.19 ECCLES, KUFFLER, AND KATZ

In 1937 Eccles returned to Australia as director of the Kanematsu Institute at Sidney Hospital, a department of diagnostic pathology whose director is expected to do research and is given financial support. Soon he had a small group. Stephen Kuffler (1913–1980) came from a landowning family in Hungary; they were wealthy but lost all in the Great Depression [53–55]. After graduation from medical school he worked as a pathologist, before hurriedly leaving Vienna. He had had some dangerous involvement in politics, which he thereafter shied away from. Unlicensed to practice medicine in England he went to Australia and was employed as a pathologist in the Sidney Hospital. Kuffler was invited for tennis on the court behind the Eccles’ house; after all he was a former Austrian tennis champion. Eccles persuaded him to join the laboratory to learn electrophysiology.

In late 1939 they were joined by Bernard Katz (1911–2003; later Sir Bernard; Fig. 1.6b), who was a Carnegie fellow [50]. Born in Leipzig, his father, a fur merchant, had come from Russia. At age nine he was at the top in the entrance examination for a prestigious gymnasium but was rejected because he was a foreign Jew. He was admitted to another school and then to the medical course at the University of Leipzig. When he received the M.D. in 1933 he was awarded a prize, given to him in private as a “non-Aryan.” He wanted to work on muscle under the

director of the Biophysics Laboratory at UCL, Archibald Vivian Hill (1886–1977; Nobel Prize 1922). Katz arrived in London in 1935 with a letter of introduction and £4 (a bit over £100 today). Within a year he and Hill published together. He received a Ph.D. at UCL and then headed for Sidney.

Eccles was working on mammalian muscle. Katz thought this far too difficult—frogs would be better. He remembered: “Stephen roaring with laughter when I showed him how to ‘take the frog’s trousers off’ ” (i.e., slip the skin off the rear legs) [55]. They placed the sartorius muscle in a chamber that had a maneuverable electrode in its floor, and stimulated the sciatic nerve. Poisoning transmission with +TC, they found areas on the muscle where they could record a localized potential change, the end-plate potential (EPP). The EPP was lengthened when the bathing solution also contained physostigmine. However, the electrical signals were complex and not easily interpreted.

World War II broke up the collaboration. Katz served in the Australian armed forces. Eccles worked on military problems. Kuffler received a research fellowship and did some further experiments on frog muscle, and in 1944 he and his Australian wife moved to the University of Chicago to the laboratory of Ralph Gerard (1900–1974).

1.20 NACHMANSOHN

Nachmansohn left for the United States before the war and in 1942 took a position at Columbia University [56]. He was working on the purification and properties of AChE, but it was difficult to get *Torpedo* for starting material. The U.S. Army asked him to investigate the effects of organophosphates on the enzyme. Some were known as potent insecticides and there was intelligence that the Germans were investigating them as potential war gases. Nachmansohn told them he would need a steady supply of electric eels from the Amazon, which an Army procurement officer dubbed “the craziest request of the war.” Nonetheless the eels came. Nachmansohn’s group showed that diisopropyl phosphorofluoridate (DFP) phosphorylates the serine on the active site of AChE and by the 1950s developed agents capable of reactivating the poisoned enzyme, such as pyridine-2-aldoxime methyl chloride (pralidoxime). Their crystallization of AChE opened the way for others, years later, to determine its structure. The reversible AChE inhibitors, such as physostigmine, bind to the active site of AChE where they are hydrolyzed just as ACh is, but at such a slow rate that they tie up the enzyme. On the other hand, the turnover of ACh by AChE is one of the fastest enzymatic conversions known.

Nachmansohn and his colleagues also showed that ACh is formed by extracts of the electric organ provided with choline and adenosine triphosphate (ATP) provided that fluoride is added to inhibit ATPase. This was revolutionary. There was no previous evidence that ATP is involved in synthetic reactions. The paper was rejected by three eminent journals. The enzyme, now called choline acetyltransferase, requires a coenzyme, which was identified by Fritz Albert Lipmann (1899–1986; Nobel Laureate 1953) as coenzyme A. Nachmansohn convinced himself that ACh is involved in axonal transmission. He convinced few others, but his dogged adherence to this mistake tends to obscure his achievements.

1.21 POSTWAR SCIENCE

World War II destroyed so much and so many, but it did increase public awareness of what scientists could do, so it was relatively easy afterward to persuade governments to keep money going to science. After a pause for economic recovery, the money began to flow and science was transformed. Figure 1.7a shows a U.S. example: the growth of the budget of the National Institute of Neurological Diseases and Stroke (NINDS). The growth in dollars is almost exponential, but the picture is altered when corrected for the decline in the purchasing power of the currency (Fig. 1.7a). The money fueled an enormous growth in the number of scientists (Fig. 1.7b and in the number of papers published on the actions of drugs on synapses (Fig. 1.7c). The American universities expanded to cope with the student veterans,

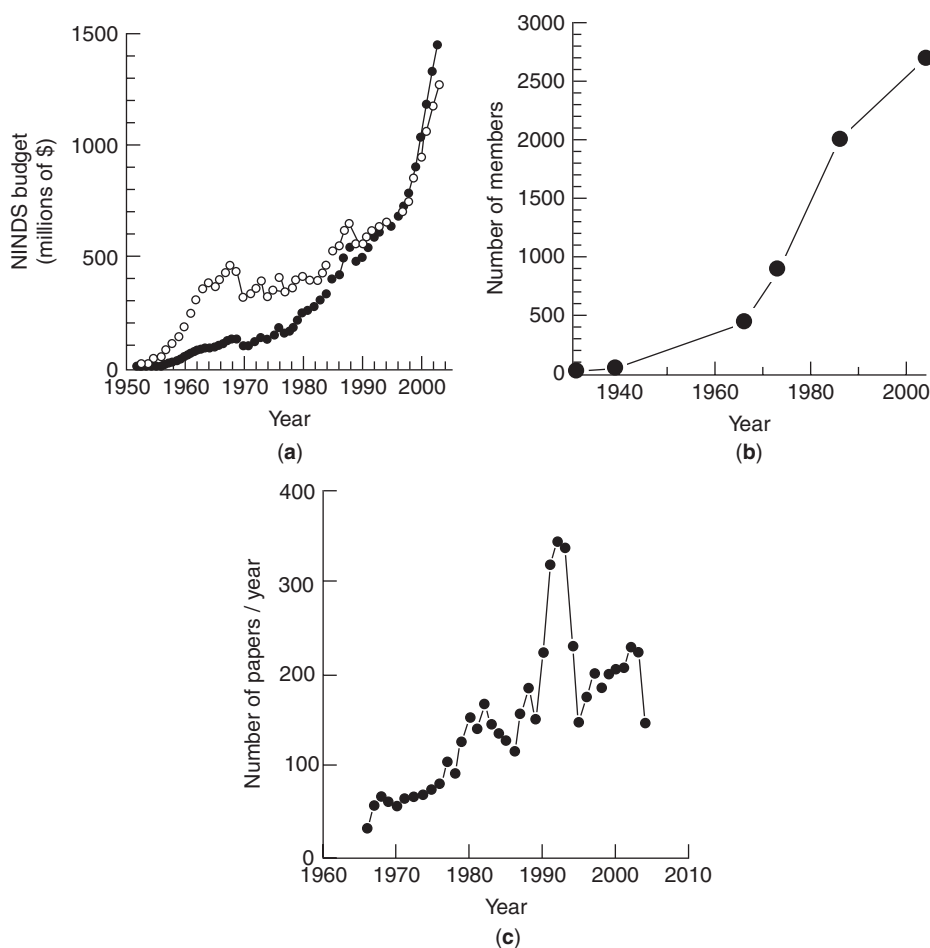


Figure 1.7 Some measures of research in neuropharmacology. (a) The budget of the NINDS filled circle (●); The budget corrected for the buying power of the dollar in 2000 (o) open circle in 2000. (b) The membership of the British Pharmacological Society. (c) The number of papers cited in the Ovid database in the category of drugs acting on synaptic transmission.

whose tuition and living expenses were paid for by the G.I. Bill, which surely must rank among the wisest legislations ever enacted.

1.22 BOVET

During the war +TC came into use to relax patient's muscles during surgery. Less anesthetic is needed for analgesia than for muscle relaxation so with +TC there is less toxicity. +TC has side effects, such as histamine release, so synthetic blocking agents were developed: some act like +TC and others act like nicotine, first stimulating and then blocking. A leader in the synthesis and testing of these compounds was Daniel Bovet (1907–1992; Nobel Laureate 1957). A native of Switzerland, he worked in France until 1947 when he and his wife, the pharmacologist F. Bovet-Nitti, moved to a new laboratory at the Istituto Superiore de Sanità in Rome. Bovet had a genius for thinking like a receptor, detecting the key operational parts of a complicated molecule such as +TC, and then synthesizing simpler compounds targeting the same receptor. One of his +TC substitutes is succinylcholine, which is hydrolyzed rapidly by ChE, so its blockade is readily reversible. He also pioneered in the development of histamine blockers and of antagonists for adrenergic receptors.

1.23 ION CHANNELS

Alan Lloyd Hodgkin (1914–1998; later Sir Alan) and Andrew Fielding Huxley (later Sir Andrew) showed how the squid giant axon generates action potentials by changes in Na^+ and K^+ permeabilities triggered by changes in membrane potential. The permeabilities are little changed by low temperature, which suggests that the ions flow through channels in the membrane. The Na^+ channel is blocked reversibly by local anesthetics and irreversibly by toxins such as tetrodotoxin, from the Japanese puffer fish, *Spherooides rubripes* [57]. The toxins were used to label the protein of channels for isolation and eventual cloning. Toxins that target the K^+ channel were discovered much later.

1.24 SOUP, NOT SPARKS

After the war Eccles wrote several influential reviews on the mechanism of synaptic transmission [58]. He conceded that neuromuscular transmission might have a chemical component but was adamant that the synapses in the CNS were electrical, both excitatory and inhibitory. As Kuffler said about him years later, “he has often been wrong, but only about important things” [54]. Chemical transmission at fast synapses would be on firm ground only when the electrophysiologists convinced themselves that it is so—until then the “soup” versus “sparks” argument would go on interminably.

The way was opened by Gerard and his colleague Gilbert Ling, a step that Gerard always downplayed because “techniques are not very important” [59]. They pulled glass microelectrodes by hand, filled them with half-strength Ringer, and manipu-

lated the tip toward a frog muscle fiber. Abruptly the pipette tip became negative to the external solution. They were measuring the resting potential of the cell. Glass fits comfortably with cell membranes, which reseal around the electrode after penetration. They could not measure action potentials, because their microelectrodes had such a high resistance that the time constant of the input stage of their electronics was too slow to follow a fast event. Hodgkin and William L. Nastuk (1917–1965) filled microelectrodes with 3 M KCl solution and used an amplifier input circuit able to cope with a high input resistance to record action potentials from skeletal muscle fibers [60]. Soon plans for making electromechanical micropipette pullers were published, then machines became commercially available, and the game was wide open.

Katz brought his Australian bride to London in 1946; he had been appointed Henry Head Fellow of the Royal Society. When the microelectrode flashed onto the scene, he returned to the neuromuscular junction, working with a graduate student, Paul Fatt. In +TC when the microelectrode was at the end plate and the nerve was stimulated, they recorded the EPP [61]. Raising the +TC concentration diminished the EPP; the drug was making less nicotinic acetylcholine receptor (nAChR) available. Anti-AChEs elongated the EPP. Using a second microelectrode to change the muscle membrane potential at the end-plate region, they showed that no EPP is seen at about 0 mV and that when the inside of the fiber was made positive the EPP was hyperpolarizing. This is because the ACh opens ion channels at the end plate that permit both Na^+ and K^+ to flow though, bringing the potential to about 0 mV, which is known as the reversal potential. The reversal was the conclusive evidence that at the neuromuscular junction (NMJ) transmission is chemical, not electrical. When ACh binds to the nAChR, it opens a chemically gated ion channel.

They then discovered small, randomly occurring depolarizations at the end plate. The depolarizations were eliminated by +TC and increased in amplitude and lengthened by anti-AChEs. They are miniature end-plate potentials (MEPPs). The ACh is released in packets or quanta of thousands of ACh molecules. Quanta may have been an unfortunate name, because it suggested to some that the packets contain a fixed number of transmitter molecules. In fact, the number can be varied over a wide range [61, 62]. The EPP is generated by the almost synchronous release of hundreds of quanta. The discovery of the MEPPs coincided with the development of tissue preparation techniques that permitted electron microscopists to see cell membranes as well as the vesicles in the motor nerve ending and in other chemical presynaptic endings.

Nastuk filled a microelectrode with ACh^+ and ejected graded amounts by adjusting electric current flow—iontophoresis [63]. ACh applied to the end-plate region opens the ion channels, but much higher concentrations are required at other parts of the muscle membrane, so the nAChR is concentrated at the end plate. Two ACh molecules usually bind to the nAChR to open the ion channel. The channel opens for a mean duration of 1 ms, determined first by statistical analysis of ACh “noise.” Other agonists open channels for different durations, depending on how rapidly they come off the nAChR. Erwin Neher and Bert Sakmann developed the patch technique to see individual channels opening. They started by observing nAChR channels on frog muscle fibers away from the end plate, where far fewer are found per unit area. They shared the Nobel Prize in 1991. Katz and Steven Thesleff showed that steady application of ACh results in a diminishing end plate current [64].

The nAChR is becoming desensitized. Other workers showed that in the desensitized receptor the affinity for ACh is increased.

1.25 ACTIONS OF +TC

+TC blocks ACh binding to the nAChR. But this is not the end of the story. The extent of the block of the nAChR by +TC varies with the potential across the end-plate membrane [65]. Part of the blocking action occurs because the drug enters open nAChR channels and plugs them. The amount of the charged drug entering the channel depends on the potential across the membrane. +TC also increases quantal output from stimulated motor nerve endings [66]. There are nAChRs on the presynaptic terminal; ACh and other agonists decrease quantal output and reduce the amount of ACh loaded into recycling vesicles [62]. There are receptors for a number of other transmitters and hormones on the motor nerve terminals [67].

Katz's papers are models to emulate: lucid, effective choice of short words and stripped of excess verbiage. His "perceptiveness in distinguishing the important from the unimportant was legendary" [50]. Such abilities were not always agreeable to his students; "there are many stories of experiences, sometimes quite traumatic, of presenting to him the first draft of a paper." He was careful and critical and eminently fair minded.

1.26 SYNAPTIC VESICLES

Synaptic vesicles containing ACh were isolated from mammalian brain and from the electric organ [68]. Victor P. Whittaker and others showed that the electric organ is a rich source and the vesicles unusually large. They found that along with the ACh the vesicles contain one ATP per four AChs and both are released by exocytosis. ATP itself is a transmitter at other junctions [69]. Studies on the electric organ elucidated the mechanism for loading ACh into the vesicles and showed that there are distinct pools of vesicles in the nerve endings [62, 68].

The most potent toxins known are made by *Clostridium botulinum*. A mouse has a 50 : 50 chance of being killed by an injection of 18 million toxin molecules [67]. They kill by blocking neuromuscular transmission. The toxins bind specifically to motor nerve endings and inject an active fragment into the cytoplasm. Transmission at other synapses is blocked if the active fragment is injected presynaptically. The toxins are Zn^{2+} -containing enzymes that target proteins involved in quantal release. Different bacterial strains produce different toxins that target different proteins, which enabled some of the proteins essential for exocytosis to be identified. Who then would have thought that these toxins would be used in huge amounts in cosmetic pharmacology?

1.27 CLONING THE nAChR

Electric organs are a splendid source of nAChR. It is made up of four subunits, and the first 54 amino acids in each of the chains were determined, a painstaking job with

the techniques available at the time. Shosaku Numa (1929–1992; Fig. 1.5d) and his group at Kyoto University prepared messenger ribonucleic acid (RNA) from electric organ and from 2.4 μg produced a complementary deoxyribonucleic acid (cDNA) library with about 200,000 clones [70]. They made small DNA probes for parts of the α -chain and then used them to search the library. It was a race of the kind seen more and more frequently since money expanded science: Laboratories in Britain, France, and the United States were working on the same lines. Numa's laboratory ran on 16-h days [71]. They first determined the composition of the α -unit; there are two of these in each nAChR, and they have ACh binding sites. Then they addressed the other three chains. The chains can reassemble spontaneously into functioning receptors. Other nAChRs are made up of different combinations of subunits. This was the first dazzling demonstration of the power of molecular neuropharmacology that figures so prominently in the rest of this book.

1.28 HOW CAN CHEMICAL TRANSMISSION BE SO FAST?

The venom of the Formosan snake, *Bungarus multicinctus*, contains a polypeptide, α -bungarotoxin, that binds irreversibly to the nAChR. The density of the nAChRs at the NMJ was measured with the electron microscope by labeling them with radioactive α -bungarotoxin. The density of the AChE was measured by reacting them with radioactive DFP [72]. Much of the AChE is on a fibrous network in the synaptic cleft. These densities, the rate constants for the reactions, an estimate of the number of ACh molecules in a quantum, and the diffusion equations were the raw material for a mathematical model of the generation of an MEPP [73]. When ACh is released from the vesicle into the narrow synaptic cleft, the local concentration is high. It diffuses across the gap saturating first the AChE in its path and then the nAChRs immediately opposite the release point. The remaining ACh diffuses along the end plate, combining with additional nAChRs to open more channels. The mean time for ACh to come off of the nAChRs is 1 ms. The nAChR is a low-affinity receptor, but because release is quantal, it is exposed to high concentrations of ACh, which it binds and then releases rapidly. Almost all of the ACh released from the nAChR is hydrolyzed by AChE before it can rebind.

1.29 ECCLES AND CENTRAL SYNAPSES

Eccles and his colleagues had been inserting fine metal electrodes into the cat spinal cord to record extracellularly from the cell bodies of motoneurons, so his group was ready to try glass microelectrodes. Astonishingly the submicroscopic tips survive being pushed through centimeters of spinal cord. They recorded intracellularly from their first motoneuron in June 1951 [34]. Activating a monosynaptic reflex by stimulating sensory axons from muscle stretch receptors, they recorded transitory depolarizations in the motoneurons. The depolarizations were named excitatory postsynaptic potentials (EPSPs). The more intensely the sensory nerve was stimulated, the larger the EPSP. When the EPSP reached a threshold level, the motoneurons fired an action potential. Next Eccles and his group inserted double-barrelled microelectrodes: one barrel to record potential changes and the second to pass a

current to set the potential of the motoneuron's membrane. Just as at the end plate, the EPSPs became hyperpolarizing when the inside of the cell was electrically positive. The reversal potential is about +3 mV. From the moment he first saw reversal the most ardent "spark" became an enthusiastic salesman for "soup."

Stimulation of an inhibitory pathway produced a slight hyperpolarization with a reversal potential of about -80 mV, an inhibitory postsynaptic potential (IPSP). By using a double-barreled microelectrode to ionophore ions into the motoneuron cell body, they showed that the reversal potential shifted to a more depolarized level after the Cl^- concentration was elevated, so the ion channel opened during inhibition is permeable to Cl^- . Hitherto they had thought that this inhibitory pathway was monosynaptic, but now they found an inhibitory interneuron, so the same cell was not both excitatory and inhibitory. They also discovered a presynaptic inhibitory mechanism which decreases the amount of transmitter released by an excitatory nerve ending.

Eccles shared the 1963 Nobel Prize with Hodgkin and Huxley. After 26 years of intense, driving, single-minded intracellular work on neurons he finally enjoyed 22 years of retirement in the Swiss mountains, working at a somewhat less intense pace on theory.

1.30 ADRENERGIC TRANSMITTERS IN CNS

Clearly there must be additional synaptic transmitters in the mammalian CNS. Obvious possibilities were adrenaline and noradrenaline. In 1954 Vogt demonstrated that noradrenaline is also found in the brain. The Nobel Prize in 1970 was shared by Ulf von Euler (1905–1983) and Julius Axelrod (1912–2004) for work on adrenergic systems. What a study in contrasts. Von Euler's father and godfather were Nobel Laureates in Chemistry and his mother was a noted botanist. He studied medicine at the Karolinska Institute; four years after matriculation he was an assistant professor of pharmacology. He worked in England with both Dale and A. V. Hill and also in Belgium and Germany. He became a professor at the Karolinska in 1939. Axelrod was born to poor parents in New York City [74]. He obtained a bachelor's degree in chemistry at the City College of New York, which at that time had no tuition charges. Graduating in 1933, at the nadir of the depression, he was rejected by medical schools so he worked as a lab assistant, a chemist in industrial hygiene where he lost an eye in a lab accident, and then in a research division of NYU. Along the way he earned an M.A. at night. At NYU his boss was Bernard B. Brodie (1907–1989): "generally called Steve Brodie. This referred to a saloon keeper named Steve Brodie, who at the beginning of the previous century had jumped off the Brooklyn Bridge to win a bet" [74a]. Brodie turned Axelrod onto the joys of research. In 1949 they moved to the newly established National Heart Institute in Bethesda. Axelrod was appointed head of pharmacology there in 1955, the same year he received a Ph.D. degree from the George Washington University.

Both von Euler and Axelrod measured amines by their fluorescence and put radioactive labels on compounds to follow their movements and metabolic transformations. Von Euler found that noradrenaline is packaged in large granules in adrenergic nerve endings, analogous to the smaller ACh-containing vesicles

at cholinergic endings. Axelrod showed that noradrenaline is broken down by monoamine oxidase but that most of the noradrenaline released from adrenergic neurons is transported intact back into cells—a new and unexpected mechanism for terminating transmitter action. The transmitter taken back into the nerve terminal is repackaged and released again. The uptake is blocked by cocaine, which accounts for its ability to potentiate sympathetic stimulation.

In the late 1940s numbers of derivatives of iminodibenzyl were synthesized and tested for their effects. A few with sedative properties were used in clinical trials. One of these, imipramine, had no quieting effect on agitated patients but by lucky chance was found to benefit depressed patients. It became the model compound for the tricyclic antidepressants. Axelrod showed that they block the reuptake of noradrenaline.

1.31 CARLSSON

Arvid Carlsson (Nobel Laureate 2000) was working on Ca^{2+} metabolism. At age 32 he applied for an associate professorship but was rejected on the ground that his subject was at a dead end. Stimulated so forcefully to shift fields, he went to work with Brodie for five months in 1955. When Carlsson arrived in the laboratory they were investigating 5-hydroxytryptamine (5-HT). It had been discovered in serum and in the gut; now they found it in the brain. They measured it with the spectro-photofluorimeter, which they had devised for detecting amines in biological samples.

They were also studying reserpine, an alkaloid from *Rauwolfia serpentina*, a shrub found in the Indian subcontinent. It had been used for centuries in Indian medicine but had just been introduced in the west for the treatment of hypertension. Its adverse reactions were sedation and, less commonly, psychotic depression. Brodie and his collaborators found that reserpine depleted the brain of 5-HT. Back in Sweden Carlsson and his collaborators showed that reserpine depleted brains of noradrenaline and that after treatment stimulated sympathetic postganglionic axons released less transmitter.

They wanted to see whether the reserpine effect could be reversed by restoring catecholamines to normal levels. Catecholamines do not penetrate the blood–brain barrier, so they decided to give a precursor that can penetrate, DOPA (3,4-dihydroxyphenylalanine). DOPA put reserpine-treated rabbits back on their feet in no time. When they measured the noradrenaline in these brains they were astonished to find it still was low. Reserpine is antagonized by monoamine oxidase inhibitors, so they reasoned that DOPA must be converted to an amine. The first step in the pathway between DOPA and noradrenaline is dopamine. Dopamine had never seemed of interest because it has little effect on tissues innervated by the autonomic nervous system. They developed a method to measure dopamine. In the brain there is more dopamine than noradrenaline, and dopamine disappears with reserpine treatment and is restored by DOPA. The effects of reserpine treatment resemble those of the Parkinson syndrome, and they found that normally dopamine is in high concentration in the basal ganglia, so they suggested that DOPA might be useful in treating the disease.

Flushed with success, Carlsson went to London in 1960 to present at a symposium. “The central figure was Sir Henry Dale, a Nobel Laureate aged 85

but still remarkably vital. He dominated the scene, and the participants, many of whom were his former students, treated him with enormous respect, like school children their headmaster, although many of them had indeed reached a mature age." Carlsson's conclusions were summarily dismissed. "Dale expressed the view that L-DOPA is a poison, which he found remarkable for an amino acid" [74a]. Dale's group pitched in to dismiss his work with equal certainty. How Carlsson must have enjoyed recounting this in his own Nobel lecture.

Carlsson and his co-workers devised histochemical methods to see precisely where catecholamines are in the brain. Within a few years they localized dopamine, noradrenaline, and 5-HT. At the next major meeting, in 1965, there was no argument whether or not these amines were important in the brain. Other investigators then carried into the clinic the role of the loss of dopamine in Parkinson's and its restoration by L-DOPA.

As we have seen, the tricyclic antidepressants, such as imipramine, were discovered in the 1950s and in the early 1960s were found to block noradrenaline uptake into nerve terminals. In the late 1960s Carlsson and collaborators found that they also blocked 5-HT uptake and developed selective inhibitors for this transporter. The best known is fluoxetine (Prozac).

1.32 SECOND MESSENGERS

Although many transmitters open ion channels, there must be other ways for them to act on target cells. Adrenergic transmitters or hormones alter cell metabolism, for instance, stimulating the liver to break glycogen down to glucose. Carl Ferdinand Cori (1896–1984) and Gerty Theresa Cori (1896–1957; Nobel Laureates 1947) elucidated the pathway by which glycogen is broken down to glucose. The enzyme phosphorylase is the rate-limiting step in glycogen breakdown. Earl W. Sutherland (1915–1974; Nobel Prize 1971) found that adrenaline increased the activity of phosphorylase in homogenates of liver cells. These homogenates also contained an enzyme that brought phosphorylase activity back to baseline without significantly altering its molecular weight. This enzyme inactivated phosphorylase by removing phosphate. In liver slices exposed to adrenaline phosphorylase becomes labeled with radioactive phosphate. The enzyme is turned on by being phosphorylated and turned off when the phosphate is removed.

Phosphorylase was not activated by adding adrenaline to homogenates. But when they also provided ATP and Mg^{2+} to the homogenates, adrenaline activated phosphorylase. They centrifuged the homogenate and worked with the soluble fraction, which contains the phosphorylase. Adrenaline did nothing. The crucial experiment was to resuspend the particulate fraction in ATP and Mg^{2+} and then expose it to adrenaline. This suspension contained a heat-stable substance that activated phosphorylase when added to the soluble fraction. The activating factor was precipitated by Ba^{2+} , so it was probably a phosphate compound. With the available techniques, it promised to be a long job characterizing the activator from the trace amounts formed by the particulate fraction. They showed that it was an adenine ribonucleotide. Sutherland wrote to a friend telling him what he knew of the properties of his activator. The friend recalled a description he had received from another investigator of a derivative produced when ATP is digested in barium

hydroxide. The properties seemed identical. This made it much easier to characterize the activator as adenosine 3',5'-monophosphate (cAMP).

Hence, adrenaline acts on a receptor on the cell membrane. This activates the enzyme adenylyl cyclase, so cAMP is formed. It acts as a second messenger that activates the kinase that activates phosphorylase. The cAMP is destroyed by phosphodiesterase, which is inhibited by alkaloids such as caffeine and theophylline. Sutherland's discoveries flung open the door for the enormous quantity of work that still continues on cellular signaling systems. An offshoot was the characterization of muscarinic ACh receptors, the identification of their second messengers, and establishing that these messengers could open some ion channels and close others. Trying not to move too far from the past, I shall merely note that the Nobel Prizes to Edmond H. Fisher and Edwin G. Krebs in 1992, Alfred G. Gilman and Martin Rodbell in 1994, and Arvid Carlsson, Paul Greengard, and Eric R. Kandel in 2000 were all for work building brilliantly on Sutherland's.

1.33 AMINO ACID TRANSMITTERS

Some Crustacean muscles have a dual innervation: excitatory and inhibitory. Neither excitation nor inhibition is affected by adrenergic or cholinergic drugs. Picrotoxin, a convulsing drug isolated from the seed of *Anamirta cocculus*, a climbing shrub from southeast Asia, blocks the effects of stimulating the inhibitory axon [75]. By the late 1950s such preparations could be ordered from the catalog, because enough money was going into biomedical research to support chemical companies specializing in the research market. The active part of this preparation, which has a difficult chemistry, is picrotoxinin (Fig. 1.2). Other workers had been looking in brain homogenates for small molecules that might be transmitters. One possibility was the unusual amino acid γ -aminobutyric acid (GABA). It inhibited excitation and its action was blocked by picrotoxin.

What about the excitatory transmitter for crustacean muscle? A Columbia medical student spent a summer seeing which amino acids caused the muscles to contract, finding that glutamate was the by far the most effective [76]. This was one of the first steps in proving that glutamate is the excitatory transmitter. Synapses in the mammalian CNS were investigated by ejecting test chemicals by iontophoresis [34]. Glutamate depolarized motoneurons. At first it was thought that this action might be nonspecific, but its identification as a transmitter was supported by the crustacean results. Aspartate is also an excitatory transmitter in the CNS. Both GABA and glycine are inhibitory transmitters in the vertebrate CNS.

1.34 KUFFLER

One of the laboratories working to expand the list of transmitters was Kuffler's [53–55]. He liked to shift preparations every few years, picking a new one that seemed promising and that tested his dissecting skills — an approach that would not survive today's grant evaluation system. They studied Crustacean stretch receptors and showed that they receive an inhibitory innervation from the CNS. The inhibition was blocked by picrotoxin and mimicked by GABA. They fractionated CNSs from 550

lobsters and tested the fractions for inhibitory molecules [77]. By far the most potent was GABA, which they then showed was much more concentrated in inhibitory than in excitatory axons. They worked on the snake neuromuscular junction, treating the preparation with collagenase and then pulling the nerve terminals away. The muscle fiber was covered with oil and droplets of solution slid onto the naked end plate. They determined that somewhat fewer than 10,000 ACh molecules were needed to generate a MEPP [78]. Kuffler's last contribution was the discovery of a "late slow" EPSP in postganglionic cells in frog autonomic ganglia produced by the release from the presynaptic neurons of a peptide resembling luteinizing hormone releasing hormone. This was one of the first demonstrations that more than one transmitter can be released at synapses. Further work revealed how complicated transmission is in the autonomic ganglia, with a series of EPSPs with different time scales and an IPSP as well and, of the importance of peptides released at synapses.

Kuffler had learned the lesson of Dale's group at the NIMR: how effectively work on the nervous system could be done by a closely interacting combination of specialists—physiologists, pharmacologists, anatomists, and biochemists—all backed by a first-rate technical staff. In 1959 Kuffler and nine colleagues established a section in the Department of Pharmacology at the Harvard Medical School. The chair was Otto Kraye (1899–1982). Kraye was not Jewish but left Germany when he rejected a chair from which a Jew had been ejected. After Kraye died Kuffler's section became the Department of Neurobiology at Harvard. His admiring colleagues regarded the establishment of the new department as a miraculous navigation of the shoals of academic politics. It became a model for many others around the world. Kuffler was skilled at keeping a group of highly distinguished and hard-driving researchers at peace with one another. Humor was his main weapon. His colleagues restricted him to "two puns a day," but this rule was frequently violated.

1.35 END OF THE ERA

Dale left the laboratory after receiving the Nobel Prize. When Wellcome died, Dale was named in his will as one of the five trustees for the Wellcome Trust [37]. The other scientific trustee was Elliott, who had become professor of medicine at UCL. Two years later Dale became the chairman of the Trust. It was a difficult and challenging job. Wellcome left everything to the Trust, which was charged with running the company, dealing with his vast collections, and supporting research. The will was lengthy and detailed but was vague on crucial points and even self-contradictory. Moreover, the business was not what it had been; Wellcome had paid it too little attention in his last years. The death duties were enormous. The trustees took the hard decision that they had no money for science—first they must pay off the duties and invest to restore the company's health. In the foundation's first 20 years only £1 million was disbursed for research. The company was saved by its U.S. branch, which had strong research—Gertrude B. Elion (1905–1998) and George H. Hitchings (1918–1999) were Nobel Laureates in 1988—and excellent management. After Dale retired as a trustee on his 85th birthday, the Trust was directed by a series of able men, who continued to plow profits back into research. It worked. By 1991 the Wellcome Trust was disbursing over £100 million a year to support scientific research. Dale died eight years after his retirement, at age 93.

Feldberg, like Kuffler, was known for his sense of humor. His after-dinner speeches to the Physiological Society were classics. In his nineties he was still experimenting. He permitted a group to film his work, purportedly for educational use. In truth they were antivivisection activists who reported him to the British Home Office. His license to use animals was revoked. He died soon after, having endured the very problem that had so plagued Claude Bernard a century and a half before.

1.36 CONCLUSIONS

In a little more than a century enough of the mechanism for chemical synaptic transmission was worked out to make much of neuropharmacology coherent. Most of the crucial observations came from experiments with poisons from nature. We have progressed from the observation that curare blocks transmission from motor nerve to muscle to the precise identification of the +TC binding sites on the nAChR.

My view is that the advance of science has been like the flow of water down a channel. Starting as a trickle for several centuries, swelling to a ripple in the seventeenth century, and now surging forward as a towering wave. The energy for building up this surge has come from the growth of human population and productivity, starting at about 1700 and continuing unabated to the present. I have written about the part of the scientific surge that is my subject by assigning progress to a few leaders riding on the crest of the wave, ignoring thousands of others. This is manifestly unfair, especially because many of the archetypes I have chosen were friends or acquaintances. Otto Loewi was an especially dear friend and thanks to his vivid descriptions I feel as though I have rubbed shoulders with Starling, Langley, Schmiedeberg, and others of that generation. My defense is that without archetypes you cannot convey any idea of what people actually did, what they were like, and the conditions in which they worked. I can only apologize to the multitude whose contributions have been ignored. When I studied the lives of my archetypes, the importance of things I had taken for granted became unmistakable: laboratories, money, fellowships, scientific meetings and societies, friends, students, collaborators, journals, and, at the top of the list, teachers. Which is why—again picking arbitrary examples—I have given each of these at least a nod.

The astonishing success and growth of neuropharmacology have been underwritten by enlightened governments. There is also a bleak reverse side in the story. Progress is not inevitable. German science was fostered by thoughtful and supportive governments. The Nazis distorted scientific policy for their abhorrent political aims. They discarded admirable people, crippling German science while unwittingly benefiting their foes. Some argue that scientists should stick to their laboratories and let others tend to the bigger picture. History does not agree.

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2

SYNAPTIC TRANSMISSION: INTERCELLULAR SIGNALING

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2.1	Introduction	40
2.2	Transmitter Synthesis	41
2.2.1	Amine Transmitters	42
2.2.2	Neuropeptides, Neurotrophins, and Growth Factors	44
2.2.3	Endocannabinoids	45
2.2.4	Gaseous Transmitters	45
2.3	Transmitter Packaging	45
2.3.1	Classical Transmitters	45
2.3.2	Peptide Transmitters and Growth Factors	46
2.3.3	Endocannabinoids, Purines, and Gaseous Transmitters	46
2.4	Synaptic Release	46
2.4.1	Vesicle-Dependent Release	46
2.4.2	Vesicle-Independent Release	47
2.5	Postsynaptic Receptors	48
2.6	Presynaptic Receptors	50
2.7	Transmitter Inactivation	51
2.8	Two Prototypic Neurotransmitters: Glutamate and Dopamine	52
2.8.1	Glutamate	53
2.8.2	Dopamine	54
2.9	Summary	56
	Acknowledgment	56
	References	56

2.1 INTRODUCTION

The “neuron doctrine” embodies concepts first proposed by Santiago Ramon y Cajal, namely that neurons are discrete cellular entities that function semi-independently and that must communicate with one another in order to sustain the processing functions of the central nervous system [1]. Despite the early evidence in support of the neuron doctrine, it was unclear how neurons actually accomplished intercellular signaling.

The multipolar anatomy of neurons spawned the concept that some domains of the neuron were exclusively local to the region containing the cell body, while others could be long and extend into distant regions of the nervous system. Combined with subsequent electrophysiological studies that defined the properties of passive and active current spread in neurons, it was thought that axons were primarily responsible for “output”-like functions of neurons, while dendrites could generally be considered to be “input” zones. Moreover, the advent of ultrastructural studies enabled, for the first time, clear evidence that there were close anatomical appositions between a variety of neuronal domains. This apposition (which corresponds to Sherrington’s “synapse”) then represents the milieu in which intercellular communication occurs.

The exchange of chemical signals across the synaptic space is the major mechanism by which intercellular signaling occurs. In the early part of the twentieth century, Otto Loewi performed experiments in which he stimulated an afferent (“presynaptic”) nerve fiber and attempted to determine how that stimulation affected the target (“postsynaptic”) tissue. He demonstrated, for the first time, that a chemical, diffusible signal (in this case acetylcholine) was able to mimic the cellular effects of stimulation of the presynaptic tissue. His studies set the stage for the concept that intercellular signaling in the nervous system was chemical in nature.

We now know that there are scores of chemical entities in the brain that serve as intercellular signaling molecules [2]. Initially, a set of molecules, mostly amino acid-derived chemicals [acetylcholine, monoamines, glutamate, γ -aminobutyric acid (GABA)], were identified as neurotransmitters that were regulated, from biosynthesis to release to inactivation, via very similar basic biochemical mechanisms. Subsequently, it became clear that these biogenic amines were only one way that neurons signaled one another, and new chemical species have been identified which obey few of the extant rules discovered through the study of the classical neurotransmitters.

The question then becomes, “what is a signaling molecule and what is not?” The criteria by which neurotransmitters are identified [2] are presented here and discussed in detail in the following sections. First, a chemical must be synthesized, either enzymatically or transcriptionally, by the neuron purported to be releasing it. Second, the chemical should be released from the neuron in a manner related to its neuronal activity (e.g., stimulation of the neuron should lead to measurable release of the chemical entity). Third, the direct application of the chemical to the postsynaptic cell should produce a physiological or biochemical effect similar to that of stimulation of the presynaptic neuron believed to be releasing the molecule. Fourth, the postsynaptic cell should express receptors that are specific for the released chemical. Finally, there should be mechanisms for postutilization inactivation of the released chemical. Taken together, these bits of evidence support the notion that a chemical found in a neuron is the (or one of the) mechanism(s) by which that cell communicates with its synaptic targets.

TABLE 2.1 Chemical Neurotransmitters

Classical neurotransmitters	
Biogenic amines	Norepinephrine (noradrenaline), dopamine, epinephrine (adrenaline), serotonin, tryptamine, histamine, acetylcholine
Amino acids	GABA, glutamate, aspartate, glycine
Neuropeptide neurotransmitters	
Peptides	Neurotensin; enkephalin, endorphin, dynorphin; neuropeptide Y; cholecystokinin; tachykinins; thyrotropin-releasing hormone; vasoactive intestinal peptide
Growth factors	Brain-derived neurotrophic factor, NT-3 and NT-4, nerve growth factor, glial cell line-derived neurotrophic factor, ciliary neurotrophic factor, epidermal growth factor, insulin, fibroblast growth factor
Unconventional neurotransmitters	
Nucleosides	Adenosine, adenosine triphosphate (ATP)
Steroids	Estrogen, cortisol, allopregenalone
Gases	NO, CO
Endocannabinoids	Anandamide, 2-arachidonylglycerol

Today, we recognize a cadre of neurotransmitter molecules that fall into three general categories, including classical neurotransmitters (biogenic amines and amino acids), peptides and growth hormones, and unconventional neurotransmitters (Table 2.1). In this chapter, we will describe these basic mechanisms that are required for chemical signaling in the nervous system. Biosynthesis, storage and release, receptor activation, reuptake, and catabolism are all important parts of the life span of the neurotransmitter, and each of these steps is critical to maintaining the integrity and discreteness of chemical signaling. Perhaps most importantly, each of these steps represents levels at which individual differences in signaling, in some cases affecting behavioral function, can arise, and pharmacological strategies can be tailored to regulating each of these levels of function.

2.2 TRANSMITTER SYNTHESIS

Biosynthesis is the first step involved in the utilization of a chemical signal. For all the known chemical signaling molecules, the chemical is generated specifically for the purposes of synaptic release. Even amino acid molecules generally involved in intermediary metabolism, such as glutamate, are biosynthesized at their release zone to generate a pool of transmitters dedicated to release. Therefore, biosynthesis represents the entry point at which a molecule dedicated to release becomes initially partitioned from the surrounding biochemical environment.

2.2.1 Amine Transmitters

The most commonly studied neurotransmitters are the amino acid–derived, small-molecule (“classical”) neurotransmitters. The production of most classical neurotransmitter substances is enzymatic in nature, occurs in the cytoplasm, is regulated by neuronal activity and intrasynaptic mechanisms coupled to signal transduction pathways, and occurs in advance of demand. Activity of synthetic enzymes can be dynamically regulated on multiple time scales: from the subsecond to the seasonal. Discrete regulatory mechanisms (transcriptional and posttranslational) act to rheostatically affect production at these various time scales.

Specific enzymes are responsible for the production of each neurotransmitter species (Table 2.2); indeed, these synthetic enzymes are sometimes expressed with sufficient selectivity to permit immunohistochemical identification of neurotransmitter phenotype. For example, cholinergic neurons are uniquely identified by immunopositive labeling for the enzyme, choline acetyltransferase (ChAT), while GABAergic neurons are recognized by staining for glutamic acid decarboxylase (GAD). In other cases, however, neurotransmitters share synthetic enzymes and pathways (Table 2.2; e.g., dopamine is the precursor for noradrenaline, which is the precursor for adrenaline, resulting in partially overlapping protein expression patterns).

The classical neurotransmitters (Table 2.1) are all amino acid derived; the enzymatic steps involve the conversion of a precursor amino acid with no or low affinity for the postsynaptic receptors into an active form which can exert transsynaptic effects (Fig. 2.1). Therefore, this productive process requires sufficient enzymatic activity (related to total numbers of enzymes present and their kinetic activity), sufficient precursor availability, and the availability of required cofactors [2]. For example, the ability of tryptophan hydroxylase (Table 2.2) to generate the indoleamine intermediate 5-hydroxytryptophan (which is subsequently transformed to serotonin) is related to the enzyme’s catalytic potential, the available levels of the precursor tryptophan (which are dependent upon dietary intake), and the presence of required cofactors such as tetrahydropteridine (a hydrogen donor), molecular

TABLE 2.2 Biosynthesis of Classical Neurotransmitters

Neurotransmitter	Synthetic Enzyme (and Cofactors)
Glutamate	Glutaminase (glutamine → glutamate)
GABA	Glutamic acid decarboxylase (glutamate → GABA)
Glycine	Serine hydroxymethyltransferase (serine → glycine)
Acetylcholine	Choline acetyltransferase (choline → acetylcholine)
Serotonin	Tryptophan hydroxylase (tryptophan → 5-hydroxytryptophan) Aromatic acid decarboxylase (5-hydroxytryptophan → serotonin)
Dopamine	Tyrosine hydroxylase (tyrosine → L-DOPA) Aromatic acid decarboxylase (L-DOPA → dopamine)
Norepinephrine	Tyrosine hydroxylase (tyrosine → L-DOPA) Aromatic acid decarboxylase (L-DOPA → dopamine) Dopamine-β-hydroxylase (dopamine → norepinephrine)
Histamine	Histidine decarboxylase (histidine → histamine)

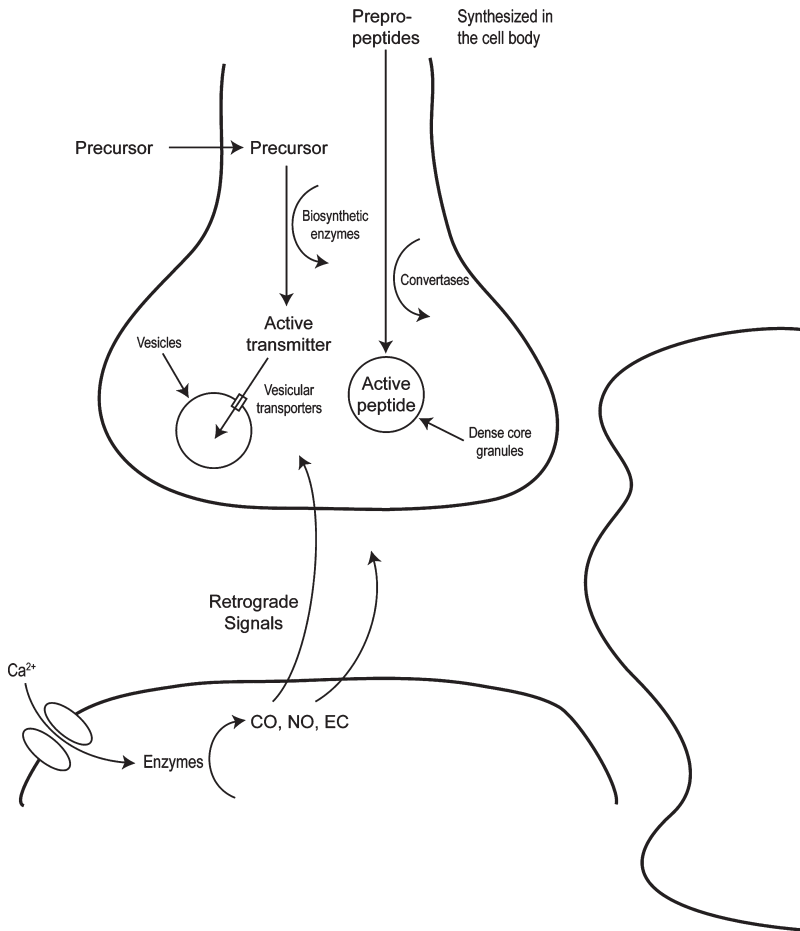


Figure 2.1 Synthesis and vesicular storage. Neurotransmitters can be biosynthesized locally at the release zone (classical transmitters, gaseous transmitters, and endocannabinoids) or produced within the cell body and transported to the release zone in a dense core granule (peptides and growth hormones). CO: carbon monoxide; NO: nitric oxide; EC: endocannabinoid.

oxygen, and Fe^{2+} [3]. As will be discussed later, the synthesis of some neurotransmitters also depends upon surrounding concentrations of the end product through a set of feedback inhibitory mechanisms.

Of course, neurotransmitter production rates are also under the control of neuronal activity, usually through elevations in cytosolic concentrations of Ca^{2+} , which in turn activate calcium-calmodulin-dependent protein kinase II (CaMKinase-II) and protein kinase C (PKC). Elevations in intracellular Ca^{2+} levels, which are associated with high levels of neuronal activity, will activate CaMKinase-II and PKC, leading to increased phosphorylation of the synthetic enzymes (e.g., tryptophan hydroxylase) and consequent change in their catalytic activity [4]. This is the primary mechanism by which neurons couple action potential activity with neurotransmitter biosynthesis rates.

Conversely, cytosolic concentrations of produced transmitter (the end product) may feedback inhibit further enzymatic activity [2]. The mechanisms for end-product inhibition are diverse but can depend upon competitive inhibition of the interaction of the required cofactors with the synthetic enzyme. Although this mechanism may be thought of as translating lower neuronal activity (and hence increases in the vesicular storage pool) of the transmitter into less synthesis, this is generally not the case. As discussed in Section 2.6, a decrease of release typically leads to greater synthesis through a release of feedback inhibition of synthesis mediated by pre-synaptic autoreceptors.

These posttranslational mechanisms represent the primary substrates by which rapid and transient changes in neuronal activity may be translated into variations in neurotransmitter production. However, there are also transcriptional changes in synthetic enzymes that are associated with long-term alterations in neuronal activity. For example, stress instantaneously activates noradrenergic neurons and increases the activity of the rate-limiting catecholamine synthetic enzyme tyrosine hydroxylase (Table 2.2) through phosphorylation-dependent mechanisms. Additionally, chronic stress can lead to an overall increase in tyrosine hydroxylase content in noradrenergic neurons: a compensation to the sustained activation of the system by the environmental challenge [5]. These changes depend upon major transcriptional regulators related to heightened neuronal activity, including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and the AP-1 complex.

Synthesis generally occurs in advance of vesicular packaging (see Section 2.3), although there is some evidence that it can also occur within vesicles (e.g., synthetic enzymes such as dopamine- β -hydroxylase can be found within vesicles and released during exocytosis; [6]). The intravesicular sequestration of the chemical signaling molecules is typically accomplished by a set of defined vesicular transporters (Fig. 2.1; also see Section 2.3).

2.2.2 Neuropeptides, Neurotrophins, and Growth Factors

Neurons release a variety of protein messengers in a typical action potential-dependent, exocytotic manner. These peptides include neuropeptides, hormones, and growth factors/neurotrophins. These peptide transmitters are frequently synthesized by cells that otherwise enzymatically generate classical amino acid-derived transmitters, a phenomenon known as colocalization.

Synthesis of these molecules is transcriptional in nature and occurs within the cell body prior to packing into dense core granules, followed by subsequent transport to the release sites (Fig. 2.1 [6]). With this in mind, the regulatory mechanisms governing peptide synthesis are slower in nature than those in control of classical transmitter synthesis, although there may still be posttranslational mechanisms that can be recruited at the synaptic terminal to rapidly increase “active” forms of the peptide [7].

Neuropeptides are frequently transcribed and translated into long, inactive (“prepro”) forms which are subsequently edited down to the final, active forms [7]. During translation, the peptides are read out into the endoplasmic reticulum, transported into the Golgi apparatus, and then packaged into so-called dense core granules. Conversion to the final, active forms of the peptide can occur within the dense core granules and involves Ca^{2+} - and pH-regulated prohormone convertases

[8]. These posttranslational events can allow intracellular events (like neuronal activity) to govern the rate of production of active peptide forms.

Neurotrophins and growth factors [e.g., brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), NT-3] tend to be larger proteins than hormones or neuropeptide transmitters. These molecules are synthesized translationally via many of the same mechanisms governing neuropeptide hormones. Some are translated into the endoplasmic reticulum and then packaged into dense core granules in the Golgi apparatus; like neuropeptides, others are not [9]. This latter pool can be moved to the release sites for signal-evoked release of the proteins.

2.2.3 Endocannabinoids

Lipid derivatives are also released in an “on-demand” fashion by cells to exert effects across a synapse; both anterograde and retrograde signaling is possible for these systems, although the latter appears to be more common (Fig. 2.1). Collectively, a group of arachidonic acid-conjugated lipids, including anandamide and 2-arachidonylglycerol, constitute the so-called endocannabinoid messengers [10].

These agents are neither synthesized in advance of utilization nor packaged into vesicles in a classical manner. The enzymes involved in the biosynthesis of endocannabinoids are Ca^{2+} regulated, and synthesis therefore occurs on demand. In subcellular domains capable of endocannabinoid secretion, a sudden increase in Ca^{2+} -mediated depolarization will activate the necessary enzymes (e.g., *N*-acyl-transferase and phospholipase D) which transfer an arachidonic acid group to phosphatidylethanolamine; the final product of this system is anandamide, which can enter the synapse through direct movement across the lipid membrane. 2-Arachidonylglycerol is synthesized from the activation of phospholipase C and diacylglycerol lipase.

2.2.4 Gaseous Transmitters

Gases, including carbon monoxide (CO) and nitric oxide (NO), are important participants in intercellular signaling in the brain [11, 12]. NO and CO can be synthesized, enzymatically, by NO synthase and heme oxygenase, respectively (Fig. 2.1). NO synthase is Ca^{2+} activated, and thus NO is synthesized on demand. Neither of these molecules is subsequently stored in vesicles, and they are capable of direct diffusion across the plasma membrane.

2.3 TRANSMITTER PACKAGING

2.3.1 Classical Transmitters

Cytosolic synthesis of transmitter is typically followed by the transport and seclusion of transmitter into vesicles (Fig. 2.1). This movement of the transmitter is mediated by a defined set of vesicular transporters, which are typically 12 transmembrane-spanning domain proteins [13] with reasonable homology to the membrane transporters (see Section 2.7). The amino acid transmitters (Table 2.1) are moved into vesicles by a set of transporters that take advantage of the electrochemical gradient across the vesicular membrane. By contrast, another set of vesicular transporters

(including those for the biogenic amines; Table 2.1) undertake the reverse transport of H^+ molecules for neurotransmitter.

Inhibition of vesicular transporters eventually leads to a disruption of action potential-dependent neurotransmitter release. For example, prolonged inhibition of vesicular monoamine transporters by reserpine leads to a dramatic and long-lasting impairment in the pool of monoamine transmitters available for on-going activity-dependent release [2]. This is because the action potential-dependent release of these transmitters generally depends critically upon this vesicular pool of transmitter (Section 2.4.1).

2.3.2 Peptide Transmitters and Growth Factors

Many of the neuropeptides that are secreted in response to action potential activity and that mediate cell–cell signaling, in this sense, are stored in vesicles called dense core granules. However, this packaging occurs after translation; most of these peptides are read out into the endoplasmic reticulum (ER) during translation [6–8]. Once in the ER, they can be moved to the Golgi apparatus for packaging into the dense core granules which are in turn transported down to the synaptic terminals or release zones. Again, posttranslational modifications are possible within the granules.

2.3.3 Endocannabinoids, Purines, and Gaseous Transmitters

Many of the most recently discovered signaling molecules, including the endocannabinoids (e.g., anandamide and 2-arachidonylglycerol), the purines (adenosine), and gaseous molecules (CO, NO), do not necessarily require vesicular storage for release. Endocannabinoids and gaseous molecules can diffuse across the membrane using passive mechanisms, while adenosine can be “pumped” out of the release zone via bidirectional nucleoside transporters [10, 11, 14]. Novel mechanisms of neurotransmitter secretion are undoubtedly possible.

2.4 SYNAPTIC RELEASE

2.4.1 Vesicle-Dependent Release

Vesicles full of transmitter are prepared for release in advance of arrival of an action potential at the release zone in order to lessen the time required to achieve synaptic release (Fig. 2.2). Vesicles can be held in place at the presynaptic release zone by a complex of proteins known as soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [15]. This complex involves protein–protein interactions between substrates expressed within the vesicular wall (e.g., synaptobrevin) and substrates within the cell membrane (e.g., syntaxin and SNAP-25). After this initial association occurs (docking), the hydrolysis of ATP by a SNARE-associated ATPase leads to destabilization of the SNARE complex and “priming” or a prefusion bridging of the vesicular and plasma membranes.

Another vesicular protein, called synaptotagmin, appears to be the Ca^{2+} -sensing protein that translates the action potential-dependent rise in cytosolic Ca^{2+} ions in the presynaptic terminal into final fusion and exocytosis [16]. Because the vesicles are already docked and primed, the influx of Ca^{2+} , which probably occurs through

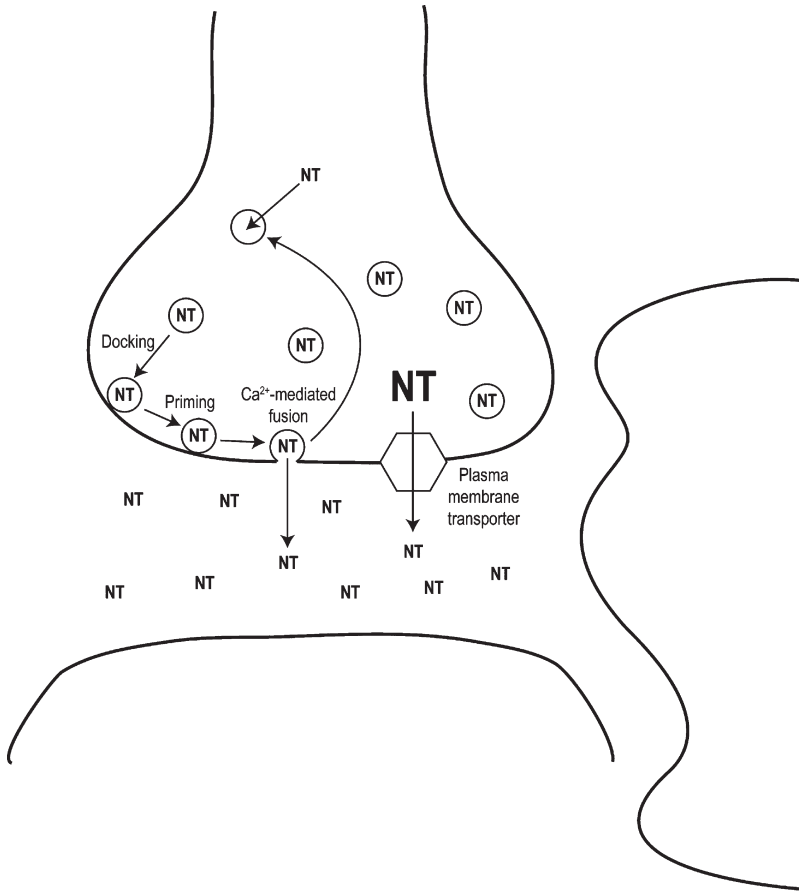


Figure 2.2 Synaptic release. Filled vesicles, which generally exist in excess of demand, are slowly moved to the release zone, where they are docked, primed, and partially fused prior to action potential generation. Influx of Ca^{2+} in response to an action potential mediates the final phase of vesicular fusion and exocytosis. Additionally, plasma membrane transporters can mediate the secretion of transmitter in a Ca^{2+} -independent fashion. NT: neurotransmitter.

channels spatially proximate to the SNARE complexes, leads to very rapid exocytosis. Therefore, there is a “microenvironment” present which is designed to minimize the time required for cell–cell signaling.

2.4.2 Vesicle-Independent Release

As noted above, some intercellular signaling molecules are capable of direct diffusion across the plasma membrane, meaning that their secretion is independent of typical vesicular storage and exocytosis. These molecules include the gaseous neurotransmitters and endocannabinoids. Their secretion is still tied to action potential generation because their synthesis is Ca^{2+} gated (Fig. 2.1).

However, even neurotransmitters typically stored in vesicles can be secreted in a vesicle-independent manner under certain circumstances. For example, the plasma membrane transporters (discussed in detail in Section 2.7) are driven, in part, by

substrate concentration gradients, which typically point from outside the cell to inside the cell. However, under circumstances where cytosolic neurotransmitters rise dramatically, for example, when vesicular transporters are disrupted and synthesis is not followed by rapid synaptic packaging, the membrane transporters can lead to an action potential-independent mode of secretion (Fig. 2.2; [17, 18]). The demonstration of this phenomenon typically depends upon pharmacological manipulations of vesicular transporter function; however, it remains possible that this form of secretion can occur under physiological conditions.

2.5 POSTSYNAPTIC RECEPTORS

Once secreted into the synaptic space, neurotransmitters must bind to and alter the activity of their receptor or effector proteins. There are four major classes of receptors that mediate the effects of release chemical substances (Fig. 2.3). These include ionotropic (ligand-gated) channels, metabotropic (G-protein-coupled) receptors, tyrosine kinase receptors, and nuclear receptors. The three former forms of receptors are membrane-bound proteins with both extracellular and intracellular domains. Alternatively, the gaseous neurotransmitters do not bind to receptors per se but rather act directly to alter the activity of target proteins, such as guanylyl cyclase in the target neuron [11]. Therefore, not all chemical signals need to act on typical “receptor” proteins.

The extracellular domains of membrane-bound receptors contain the motifs to which neurotransmitters bind in order to achieve a conformational change in the protein. In the case of ionotropic receptors, the resulting conformational change in the receptor leads to the opening of a central pore that permits the movement of cations or anions across the membrane, resulting in rapid membrane depolarization or hyperpolarization [6]. These ionotropic receptors are typically heteropentameric receptor complexes that constitute combinations of potentially large families of receptor subunits (at least 16 genes encode subunits for glutamate ionotropic receptors; at least 12 genes encode subunits for ionotropic acetylcholine receptors; etc. [6]). Ionotropic receptor subunits typically each confer a unique set of properties (neurotransmitter binding pocket, selectivity of ion permeability, allosteric regulatory sites, etc.) to the mature protein, which allows for variability in the properties of these receptors.

G-protein-coupled, seven-transmembrane-spanning domain proteins represent the most common form of receptors for intercellular signals. More than 350 separate genes in the human genome encode ligand-gated G-protein-coupled receptors [19], representing a major superfamily of proteins mediating intercellular signaling. These receptors exert their intracellular effects through activation of heterotrimeric guanosine triphosphate (GTP) binding (G) proteins; there are 35 separate genes in the human genome encoding the various subunit proteins that can enter into these heterotrimeric complexes. In total, there are four major families of complexes: the $G_{s/olf}$, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ families [20, 21]. Each family produces a different subset of intracellular biochemical events, ranging from alterations in ion channel function to regulation of lipase activity to the mobilization of kinases and phosphatases. Importantly, transmitter-dependent activation of ionotropic receptors and associated membrane currents (see above) are subject to modification by intracellular signaling

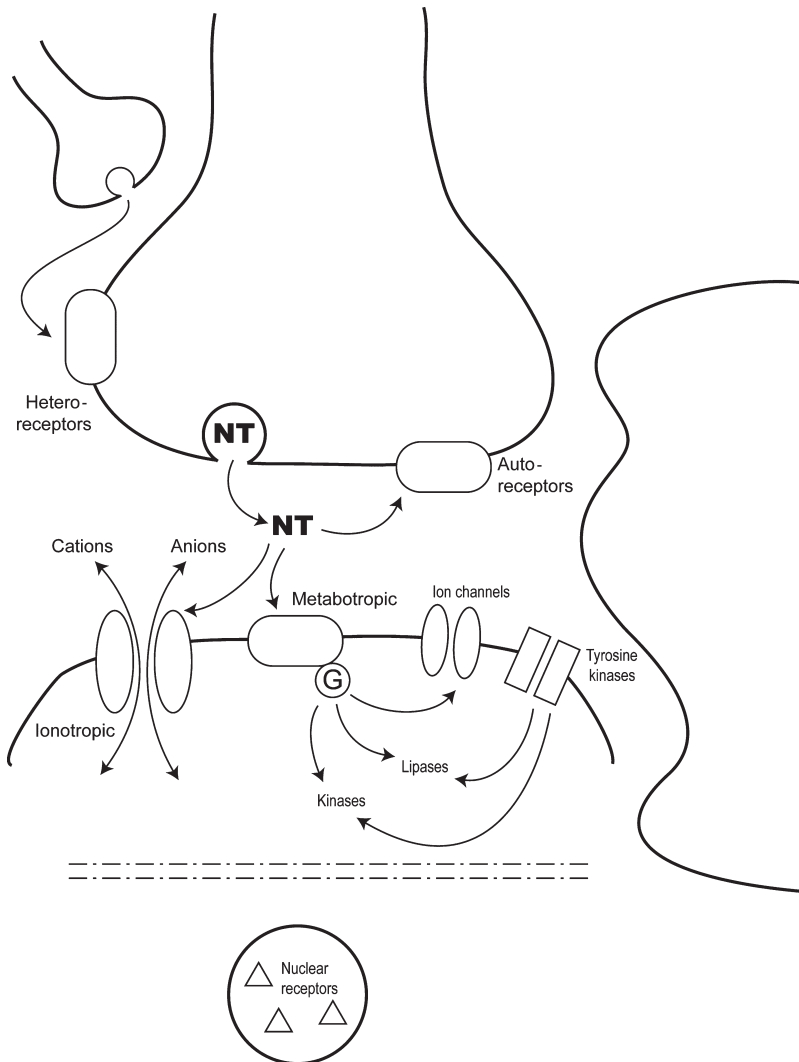


Figure 2.3 Receptors. Four major types of receptors, located pre- and postsynaptically, mediate neurotransmitter effects. These include ligand-gated ion channels (ionotropic receptors), G-protein-coupled proteins (metabotropic receptors), tyrosine kinase receptors, and intracellular nuclear receptors.

pathways, mostly through phosphorylation of the intracellular domains of the receptors or through direct gating by G proteins.

Nuclear receptors represent the signal by which steroid hormones achieve their physiological effects on cells [22]. Steroids, such as cortisol, estrogen, and allopregalone, can pass directly across cell membranes and reach their target receptors which are located within the cytoplasm. These receptors are typically immobilized by heat-shock proteins when not activated by their ligands. The interaction of the ligand with its receptor leads to a dissociation of the heat-shock protein, which in turn permits the movement of the receptor complex to the nucleus. In the nucleus, these

receptors act as transcription factors that can positively or negatively regulate gene transcription.

The final major family of receptors are the tyrosine kinase receptors [23]. These are transmembrane-spanning domain proteins that are capable of dimerization and autophosphorylation subsequent to binding by its endogenous substrates (which are typically the neurotrophins and growth hormones). Once phosphorylation of the dimer has occurred, the tyrosine kinase receptors are able to engage in protein–protein interactions that mediate their intracellular effects, such as the activation of Ras, phosphatidylinositol-3-kinase or phospholipase C.

These four major types of receptors allow for a diverse range of intracellular effects of released chemical signals. Because of the diversity of receptor types, intercellular signaling is capable of eliciting rapid changes in membrane polarization, mobilizing changes in intracellular kinase/phosphatase function, and directly affecting gene expression.

2.6 PRESYNAPTIC RECEPTORS

Most axonal terminals express receptors either for their own chemical transmitter (so-called autoreceptors; Fig. 2.3) or for the transmitter that is being released by another, nearby terminal (heteroreceptors; Fig. 2.3); these modes of transmission roughly equate to autocrine and paracrine signaling, respectively [2].

Simply, these presynaptic autoreceptors allow the releasing terminal to regulate the magnitude of its synthesis and release in a manner that stabilizes synaptic concentrations of the neurochemical within a narrow range. Therefore, these presynaptic receptors can be generally thought of as a mechanism by which feedback inhibition on neurotransmitter release is exerted by a variety of synaptically located chemical substances.

Autoreceptors typically mediate feedback inhibition on synthesis and release through a $G_{i/o}$ -coupled receptor. The binding of transmitter to the autoreceptor can produce a mobilization of $G_{i/o}$ protein activity, which in turn can activate G-protein-coupled inward-rectifying K^+ channels (GiRKs), leading to presynaptic terminal hyperpolarization [6]. Through this mechanism, increasing synaptic concentrations of transmitter translate into decreased probabilities of subsequent synaptic release events. These “release-regulating” autoreceptors represent a set of exciting targets that may represent novel methods for modulating neurotransmitter release by acting on the mechanisms designed to preserve homeostasis.

Additionally, presynaptic autoreceptors can modulate synthesis directly by decreasing cAMP concentrations and, in turn, PKA-dependent phosphorylation of the enzymatic biosynthetic machinery [2]. For example, in the dopaminergic system, cessation of dopamine release is accompanied by a dramatic increase in catecholamine synthesis that is secondary to reduced activation of presynaptic autoreceptors [24, 25]. In principle, the increase in synthetic capacity is designed to ensure that the reduction in release is not secondary to a depletion of transmitter reserve.

Other terminals may also regulate presynaptic function by releasing molecules that act on heteroreceptors. Many of these receptors (both ionotropic and metabotropic) may act to cause depolarization or hyperpolarization of the terminal, to affect phosphorylation of synthetic enzymes and/or of synaptic release elements, and so on. For example, excitatory presynaptic ionotropic acetylcholine receptors are expressed

by glutamatergic terminals, leading to a powerful control of glutamate secretion by acetylcholine [26, 27]; additionally, many of the typical autoreceptor proteins act as heteroreceptors by governing GiRK function on nearby terminals through heterosynaptic mechanisms. An increasing number of diverse mechanisms of presynaptic release modulation are becoming apparent.

2.7 TRANSMITTER INACTIVATION

Inactivation of released neurotransmitter is typically accomplished by active enzymatic biotransformation of the active signaling molecule and/or by internalization of the neurotransmitter via membrane-bound transporter proteins (Fig. 2.4). Pre- and postsynaptic neurons, as well as adjacent glial cells, are each participants in the processes of transmitter inactivation.

Intracellular uptake (internalization) of the transmitter, usually through a set of homologous 12-transmembrane-spanning domain proteins called plasma membrane transporters [28], is one mode of neurotransmitter inactivation. These transporters can be expressed by either the releasing entity or the glial cells that surround the synapse. There are molecularly unique transporter proteins that correspond to each of the major classical neurotransmitters; in fact, some transmitters (e.g., glutamate) can be taken up by multiple transporter proteins. Therefore, there is a sizable gene family that encodes for the membrane transporters.

Acetylcholine and the monoamines are largely reuptaken by the terminals that released the chemicals, while GABA, glutamate, and other amino acids are taken up by surrounding glial cells. This uptake process is sensitive to the concentration gradient of the neurotransmitter and to the availability of Na^+ . It is worth noting that these transporters may directly internalize the released chemical and/or may target a biotransformed product of the molecule because some transmitters are enzymatically modified prior to reuptake [2].

Neurotransmitters can be enzymatically biotransformed into molecules inactive at synaptic receptors, and this conversion can happen within the synapse or after internalization via a transporter (Fig. 2.4). The fate of the molecules, once biotransformed, can vary. In some cases (e.g., serotonin), neurotransmitters are catabolized into molecules that are then excreted [2]; in other words, the molecules do not reenter the neurotransmitter utilization pathways. In other cases, however, the neurotransmitter is converted into a molecule that can reenter the biosynthesis pathway. For example, acetylcholine, GABA, and glutamate are transformed either directly or through successive steps into their own precursor molecules (choline, glutamate, and glutamine, respectively), enabling the process of recycling [6]. Yet other molecules, such as peptide transmitters and growth hormones, are subjected to proteolysis in the synapse in order to eliminate or alter their further physiological effects.

For virtually all the classical neurotransmitters, a combination of biotransformation and reuptake is typical [2]. For example, GABA is taken up by glial cells and enters the “GABA shunt”, which involves successive deamination of GABA to succinic semialdehyde and amination of α -ketoglutarate to glutamate, which can then be transported back to the releasing presynaptic terminal for further synthesis of GABA [6]. On the other hand, serotonin is largely taken up by the serotonin transporter, followed by successive oxidation and dehydrogenation [3].

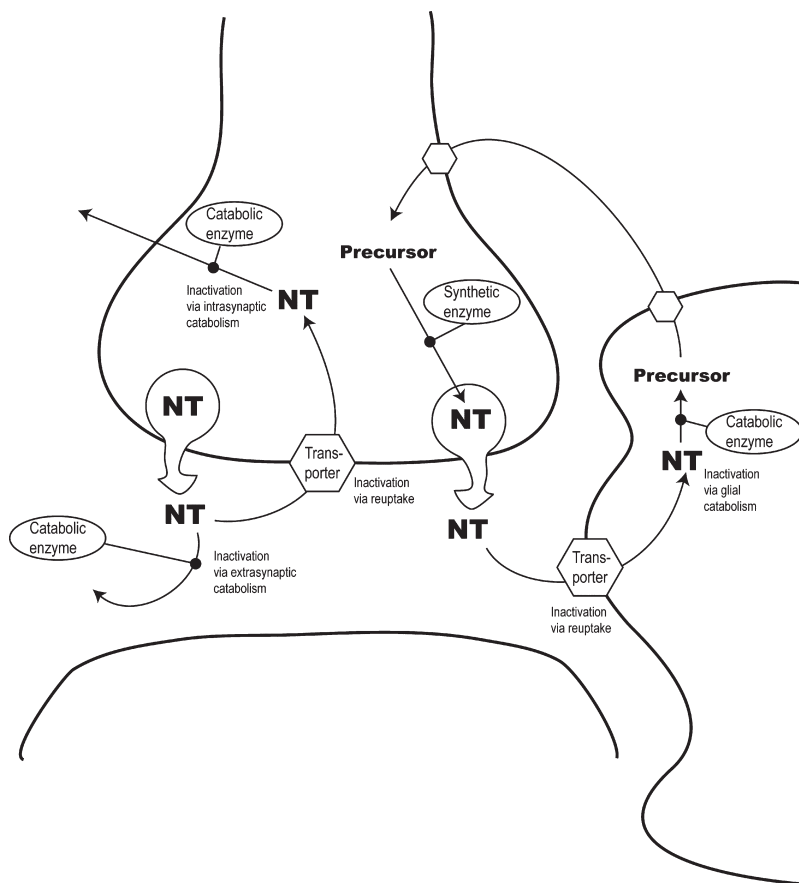


Figure 2.4 Transmitter inactivation. The inactivation of neurotransmitter typically involves reuptake and/or further biotransformation. In some cases, reuptake is into the presynaptic terminal, while in others it can be into surrounding glial cells. Biotransformation can involve the generation of a product that can reenter the biosynthesis pathway (recycling) or of an inactive molecule (catabolism).

Other chemical signals, such as the gaseous neurotransmitters, have no known inactivation mechanism [11]. The end of their physiological effects may depend simply on diffusion along their concentration gradient, away from the site of release and target action. It is notable that the lack of inactivation mechanisms technically violates the criteria for a neurotransmitter described above in Section 2.1. Obviously, the discovery of new chemical signaling molecules may require a progressive refinement of the “rules” of chemical signaling.

2.8 TWO PROTOTYPIC NEUROTRANSMITTERS: GLUTAMATE AND DOPAMINE

The previous sections outlined the general principles by which intercellular signaling is generally accomplished in the nervous system. Here, two classical transmitters,

glutamate and dopamine, will be described in greater detail in order to more fully illustrate the usual mechanisms for intercellular signaling in the brain.

2.8.1 Glutamate

Although glutamate is a common amino acid that participates in various aspects of intermediary metabolism in neurons (including as a precursor for another neurotransmitter, GABA), it also is a key excitatory amino acid neurotransmitter belonging the group of classical signaling molecules described above (Table 2.1). Despite the prevalence of glutamate and its functions, the neurotransmitter pool of glutamate is regulated according to the general principles described in the preceding sections. Figure 2.5 illustrates the mechanisms governing glutamate synthesis, packaging, release, receptor action, and inactivation.

As the principal excitatory neurotransmitter in brain, glutamate is synthesized and released by neurons whose cell bodies are found throughout the central nervous system. In these neurons, the neurotransmitter pool of L-glutamate is biosynthesized from the precursor glutamine by the enzyme glutaminase (Table 2.2, Fig. 2.5). Alternative pathways are possible, including the transamination of α -oxoglutarate [2]. Cytosolic synthesis of glutamate is followed by its packaging into vesicles via a set of vesicular glutamate transporters consisting of at least three separate gene products (vGluT1–3; [29]). The intravesicular transport of glutamate, which is stereoselective, is dependent upon the electrochemical gradient across the vesicular membrane. The filled vesicles are subsequently docked, primed, and exocytosed in response to action potential-dependent increases in cytosolic Ca^{2+} .

Once released into the synapse, glutamate is free to act on both ionotropic and metabotropic receptors, located both pre- and postsynaptically. These include the ligand-gated ion channels, termed *N*-methyl-D-aspartate (NMDA)-sensitive, kainate-sensitive, and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-sensitive glutamate receptors. Each is a glutamate-regulated, heteropentameric cation channel that is constructed from combinations of subunit proteins drawn from a family of at least 14 gene products: GluR1–7, KA1–2, NR1 and 2A–D [30]. Without a doubt, these various subunits confer individual properties to the mature subunit complex (allosteric binding sites, additional kinetic properties, etc.), so although there are three general types of ionotropic glutamate receptors (NMDA, AMPA, kainate), there may be additional diversity within each class related to subunit composition.

An additional set of at least eight gene products makes up the metabotropic G-protein-coupled glutamate receptors (mGluR1–8; [31]). This group of receptors largely signals intracellularly through either G_i (mGluR2–4,6–8) or G_q (mGluR1,5) pathways. It is the mGluR2–3 receptors that constitute the presynaptic autoreceptor for the glutamate system [32, 33], exerting feedback inhibition on glutamate release and synthesis.

After receptor activation, glutamate is inactivated by uptake, either into the presynaptic terminal or (more generally) into glia. In brain, the uptake of glutamate into glia occurs via an excitatory amino acid transporter (EAAT), specifically EAAT1 and EAAT2. Subsequent conversion of glutamate to glutamine by glutamine synthetase is followed by recycling of glutamine back to the releasing terminal for subsequent entry into the biosynthetic pathway.

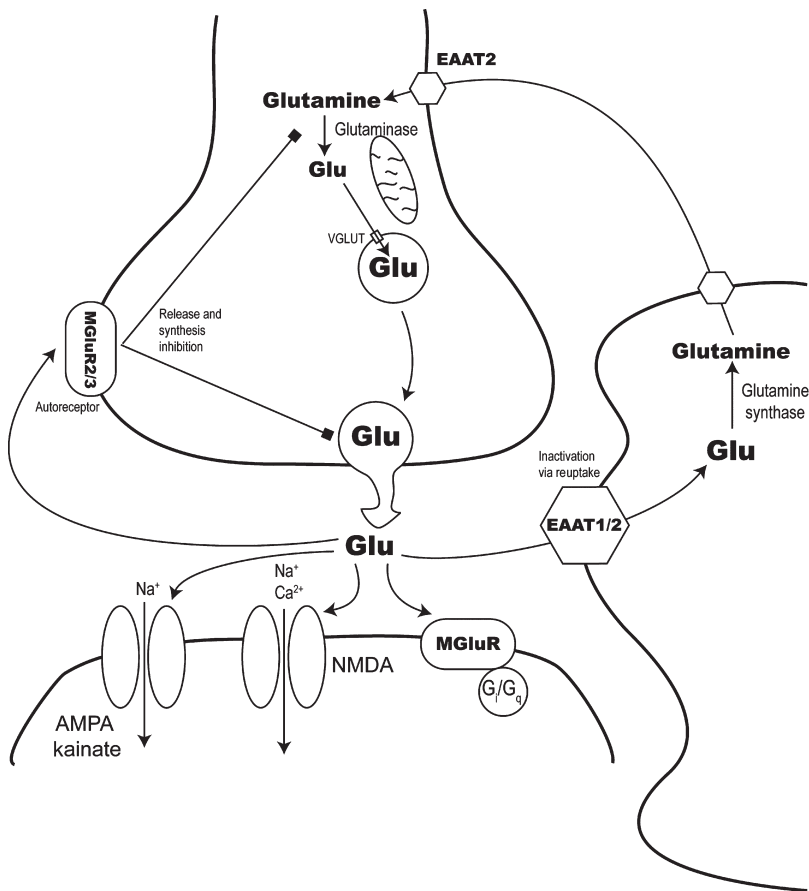


Figure 2.5 Biochemical mechanisms for glutamate synthesis, release, and disposition. Glu: glutamate; VGLUT: vesicular glutamate transporter; EAAT: excitatory amino acid transporter; MGluR: metabotropic glutamate receptor; NMDA: *N*-methyl-D-aspartate/glutamate receptor; AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/glutamate receptor.

2.8.2 Dopamine

Dopamine is a catecholamine transmitter that belongs to the group of biogenic amine molecules (Table 2.1). The neurons that make and release dopamine have cell bodies localized chiefly in the medulla, ventral mesencephalon, and hypothalamus. Figure 2.6 illustrates the biochemical mechanisms found at the synaptic terminals of these dopamine-releasing neurons that contribute to synthesis, packaging, release, post-synaptic actions, and inactivation.

Dopamine is synthesized via subsequent hydroxylation and decarboxylation steps. Tyrosine is converted into L-DOPA by tyrosine hydroxylase, and L-DOPA is sequentially converted into dopamine by aromatic acid decarboxylase (Table 2.2, Fig. 2.6). Once synthesized, dopamine is sequestered via the action of a vesicular monoamine transporter protein that successfully moves dopamine into vesicles via the reverse transport of H^+ . As with glutamate, these vesicles are then prepared for Ca^{2+} -dependent exocytosis.

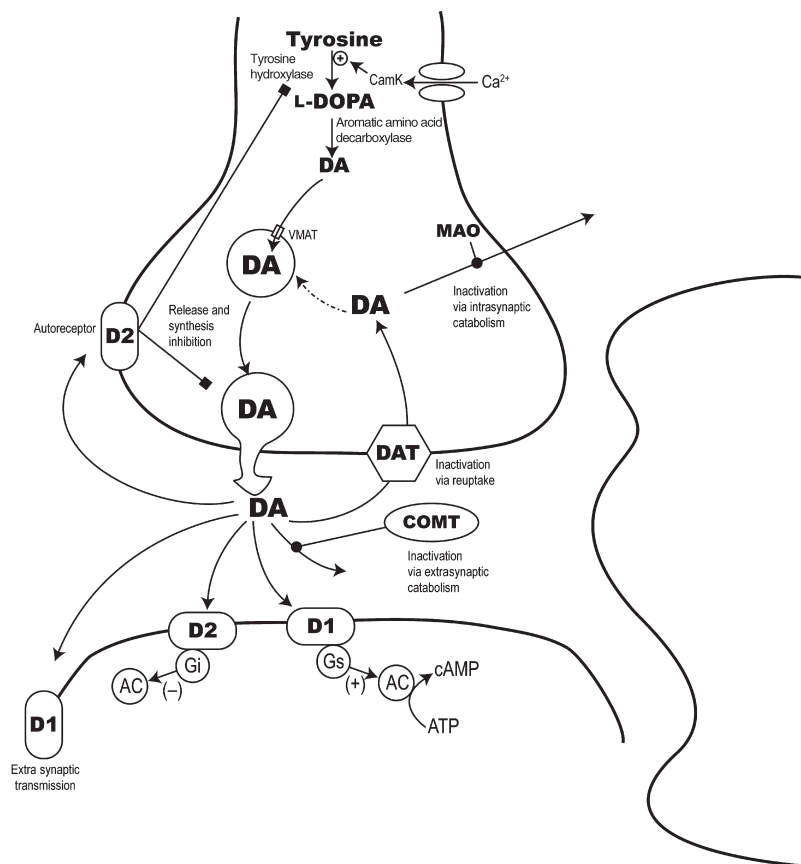


Figure 2.6 Biochemical mechanisms for dopamine synthesis, release, and disposition. DA: dopamine; VMAT: vesicular monoamine transporter; MAO: monoamine oxidase; COMT: catechol-*O*-methyltransferase; CamK: CaMKinaseII; DAT: dopamine transporter; AC: adenylyl cyclase.

Once released, dopamine acts on a set of at least five metabotropic G-protein-coupled receptors that are located both pre- and postsynaptically [34]. This family of receptors (D₁–D₅) are the mechanisms for (1) dopamine-mediated cAMP formation and phosphoinositide hydrolysis (via coupling to G_s) or (2) dopamine-mediated reductions in cAMP, opening of GiRKs and inhibition of Ca²⁺ channels (via coupling to G_i). The D₁ and D₅ receptors perform the former action, while the D₂, D₃, and D₄ receptors contribute to the latter.

Disposition of dopamine occurs via a number of routes. First, extracellular catabolism by catechol-*O*-methyltransferase (into 3-methoxytyramine) can occur; this enzyme is thought to be expressed by glia or postsynaptic neurons [35], rather than by dopamine cells themselves. Second, reuptake via dopamine transporters, followed by oxidation and methylation, is a typical means, as indicated by the high levels of the corresponding catabolites dihydroxy-*O*-phenylacetic acid and homovanillic acid in rats and humans, respectively [3].

Notably, not all routes occur equally in all brain regions. In the prefrontal cortex, one target of innervation by the dopamine neurons of the ventral mesencephalon, the dopamine transporter exists at very low levels [36, 37], meaning reuptake via this transporter contributes only minimally to inactivation, while it plays a much larger role in other brain regions, such as in the striatum. In the prefrontal cortex, therefore, extracellular catabolism is a prominent mechanism for dopamine disposition.

A final point which bears discussion is the discovery that dopamine actually exhibits higher affinity for the norepinephrine transporter than does norepinephrine itself [2]. With minimal dopamine transporter available, the norepinephrine reuptake transporters may play a significant role in dopamine disposition, a hypothesis supported by the finding that norepinephrine transporter inhibitors elicit increased synaptic levels of dopamine in the prefrontal cortex [38]. Of even greater interest, norepinephrine transporter immunoreactive cells in the prefrontal cortex do not express considerable amounts of the enzymes required to synthesize dopamine [39, 40], which is a required precursor for norepinephrine. Therefore, it is possible that noradrenergic neurons, under some circumstances, utilize dopamine taken up from the extracellular space as a precursor to produce their own neurotransmitter. New discoveries such as this clearly require an expansion of the way we think about neurotransmitter function.

2.9 SUMMARY

Although the discovery of a general set of principles describing the life span of classical neurotransmitters has been helpful in characterizing their functions as well as the modes of actions of psychotropic drugs that alter these pathways, these principles have also kept us ignorant of the way that less conventional mechanisms of intercellular signaling work in the brain. For example, the “paradigm” of action potential-dependent vesicular release does not apply to the gaseous and lipid transmitters, and inactivation mechanisms vary considerably across neurotransmitters and brain regions. The discovery of novel transmitters also allows for the discovery of novel means of chemical signaling, and it is undoubtedly the case that many new mechanisms will be found in coming years.

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3

SYNAPTIC TRANSMISSION: INTRACELLULAR SIGNALING

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3.1	G Proteins as Signal Transducers	60
3.1.1	Heterotrimeric G Proteins	60
3.1.2	Small G Proteins	63
3.1.3	Regulation of G-Protein Cycle	64
3.1.4	Alternate Roles for RGS Proteins	65
3.1.5	G Proteins and Disease	66
3.2	GPCR–G Protein Modulation of Ion Channels	66
3.2.1	G $\beta\gamma$ Signaling to Ion Channels	67
3.2.2	Other Downstream Signaling Molecules That Modulate Ion Channels	69
3.2.3	Modulation of Ion Channels by Direct Protein–Protein Interactions with GPCRs	70
3.3	Signaling Through Cyclic Nucleotide Second Messengers	72
3.3.1	Adenylyl Cyclase	72
3.3.2	Cellular Targets of cAMP	75
3.3.3	Guanylyl Cyclase	78
3.3.4	Cellular Targets of cGMP	80
3.4	Phospholipase C–, Protein Kinase C–, and Ca ²⁺ -Regulated Signaling Pathways	81
3.4.1	IP ₃ and Phosphoinositides as Signaling Molecules	81
3.4.2	Ca ²⁺ as a Signaling Molecule	83
3.4.3	DAG Activates Protein Kinase C	84
3.4.4	Action of Ca ²⁺ and Its Mediator Calmodulin	85
3.5	Protein Phosphorylation	87
3.5.1	Protein Tyrosine Kinases	87
3.5.2	Mitogen-Activated Protein Kinases	89
3.5.3	G-Protein-Coupled Receptor Kinases	90
3.5.4	Protein Tyrosine Phosphatases	92
3.5.5	Serine/Threonine Phosphatases	94
3.6	Conclusion	96
	References	96

In synaptic signaling, the response of an individual neuron is determined by its complement of receptors. As discussed in other chapters in this volume, ligand-gated ion channels mediate fast synaptic transmission between neurons. However, signaling within an individual neuron is mediated by a complex set of interactions that occur primarily as a result of G-protein-linked signal transduction. The beauty of this system is in its diversity and inherent capacity for amplification and plasticity in the modulation of many different cellular functions. The initial signal sequence in this type of signaling typically is the generation of a second messenger inside the cell that then in turn activates a number of different proteins capable of modifying cellular processes. This chapter will present examples and an in-depth discussion of these intracellular signaling pathways beginning with G-protein-linked signaling, perpetuated by G-protein-coupled receptor (GPCR) stimulation. Next, ion channel modulation via G proteins as well as direct GPCR interactions with the channels will be discussed. The long list of intracellular downstream effector pathways, including cyclic nucleotide signaling, protein kinase signaling, and calcium-regulated signaling, are also described. The final focus of the chapter is protein phosphorylation cascades, a predominant mechanism in many signal transduction schemes. It is through these complex interactions that an individual cell is able to respond to neurotransmitter stimulation and modify many of its cellular processes to contribute to the overall symphony of synaptic signaling.

3.1 G PROTEINS AS SIGNAL TRANSDUCERS

Classically, receptors bind neurotransmitter, hormone, or other ligands on their extracellular surface, thus causing a conformational change and transference of the ligand-encoded signal/message to the intracellular compartment for propagation. Many of these signal-transducing receptors belong to the GPCR superfamily. GPCRs consist of seven transmembrane-spanning α -helix bundles that snake through the plasma membrane. Mutagenesis and biochemical experiments suggest that ligand binding to the extracellular face of the GPCR changes the relative orientation of the transmembrane domains. This conformational change carries through to the intracellular loops and carboxyl terminus, thus unmasking protein interacting domains [1]. Importantly, among the protein interacting sites in this intracellular region are amino acid residues critical for the interaction with heterotrimeric guanosine triphosphate (GTP)-binding regulatory proteins, or G proteins.

3.1.1 Heterotrimeric G Proteins

G proteins consist of subunits designated α , β , and γ . Multiple isoforms, activating a variety of signaling pathways, have been discovered for each of these subunits (Table 3.1). At present, there are 23 known isoforms of the human α subunit. $G\alpha$ is localized to the plasma membrane via lipid modification, specifically myristoylation and palmitoylation [2]. These modifications assist not only with membrane trafficking of the protein but also with membrane anchoring and interactions with $G\beta\gamma$ and downstream effectors [3, 4]. The $G\alpha$ subunit is a “flexible” protein consisting of two distinct domains—a Ras-like GTPase region and a helical domain. Together, these domains form the binding pocket for GTP and guanosine diphosphate (GDP) [2].

TABLE 3.1 G-Protein Subtypes

G-Protein Family	Subtype	Consequence ^a	Tissue Distribution	Comments ^b
G_{i/o}	G _{zs}	↑ Adenylyl cyclase	Ubiquitous	1
	G _{zolf}		Brain/olfactory	1
	G _{zi1,2,3}	↓ Adenylyl cyclase	Wide	2
	G _{zoA,B}	↑ K ⁺ channel	Brain	2
		↓ Ca ²⁺ channel		
G_q	G _{qt1,2}	↑ PDE6	Retina	3
		↓ cGMP		
	G _{qz}	↓ Adenylyl cyclase	Brain/retina	
	G _{zq}	↑ Phospholipase C β1	Ubiquitous	
	G _{z11}		Ubiquitous	
	G _{z14}		Stromal/epithelial	
	G _{z15}		Myeloid	
G₁₂	G _{z16}		Myeloid	
	G _{z12}	Rho GTP exchange catalyst	Ubiquitous	
	G _{z13}		Ubiquitous	
G_β	β ₁ –β ₄ , β ₆			
	β ₅			
G_γ	γ ₁ –γ ₁₂			

^acGMP = cyclic adenosine monophosphate; PDE = phosphodiesterase.^b(1) Cholera toxin Activation. (2) Pertussis toxin inhibition. (3) Pertussis toxin insensitive.

There are 6 known human isoforms of G_β and 12 of G_γ; these subunits also contain lipid modifications for membrane targeting [5]. With relatively few exceptions, most β and γ isoforms can form functional heterodimers, together forming a structure consisting of a β propeller and an α-helical region that is far more rigid than the G_α subunit [6]. There is currently much interest in the importance of specific G_{βγ} dimers utilized within individual signaling pathways. The possibility of differential association of the α subunit with these βγ dimers adds another level of complexity to the generation of signals downstream of activated GPCRs.

The activated receptor interacts with the inactive, GDP-bound form of the heterotrimeric G protein to form the RG complex, thus inducing GDP release from the G protein and beginning the G-protein activation cycle (Fig. 3.1 [7]). It is hypothesized that the receptor–G protein contact sites are separated from the G-protein GDP binding pocket, so that the receptor must “work at a distance” to change the G-protein conformation and release the GDP [8]. Upon release of the GDP, a stable complex is formed between the receptor and heterotrimeric G protein; this complex is termed R*G. GTP then binds to the G protein, inducing a conformational change in the G_α subunit that leads to decreased affinity for the G_{βγ} subunits and the receptor. This conformational change also unmask a hydrophobic pocket on the G_α subunit that is the site of interaction with effector proteins [9]. The complex then dissociates from the receptor, releasing activated G_α–GTP and G_{βγ}, which can each interact with and regulate various multiple effector molecules before cessation of activity [7]. The G-protein signal is terminated by intrinsic hydrolysis activity of the G_α subunit, which converts the

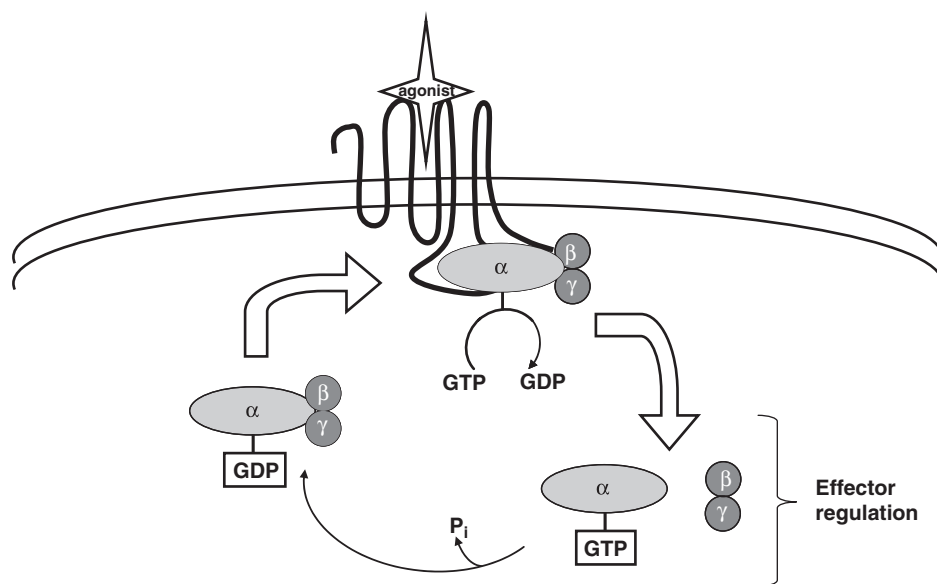


Figure 3.1 Heterotrimeric G-protein activation/inactivation cycle. In the absence of agonist, the GPCR interacts with the GDP-bound form of the G protein. Following agonist binding, GDP is exchanged for GTP and the G protein dissociates from the receptor, separating into GTP-bound $G\alpha$ and $G\beta\gamma$ subunits. Each of these subunits is capable of stimulating effector proteins. After initiating downstream signaling cascades, the $G\alpha$ subunit is turned off via hydrolysis of GTP to GDP. The $G\beta\gamma$ subunit can then reassociate with the GDP-bound $G\alpha$ subunit, thus shutting off G-protein activity and enabling reassociation with the GPCR.

$G\alpha$ -bound GTP to GDP. Association of $G\alpha$ with GDP increases the affinity of $G\alpha$ for $G\beta\gamma$. The heterotrimeric G protein re-forms and is again able to interact with the receptor.

The downstream signaling targets of all four $G\alpha$ classes have been well established and are listed, along with expression profiles, in Table 3.1; these pathways will also be covered in greater detail in subsequent sections. Briefly, G_s and $G_{i/o}$ both modulate adenylyl cyclase (AC) to alter cyclic adenosine monophosphate (cAMP) levels and affect protein kinase A (PKA) activity. G_q proteins target the phosphoinositide pathway by increasing phospholipase C (PLC) activity, which causes downstream release of intracellular Ca^{2+} and activation of protein kinase C (PKC). Activation of $G_{12/13}$ causes stimulation of specific RhoGEF proteins, which can activate phospholipase D (PLD) and other effectors. It was once believed that $G\beta\gamma$ proteins functioned solely to hold $G\alpha$ subunits in an inactive state. However, it is now known that, upon dissociation from $G\alpha$, the $G\beta\gamma$ dimer is capable of activating effector proteins as well. The known downstream effectors of $G\beta\gamma$ are protein kinases, small G proteins, AC, and ion channels, including N and P/Q-type Ca^{2+} channels and G-protein-regulated inward-rectifier K^+ channels. Through the many isoforms and potential heterotrimer combinations, the ability of a single activated G-protein subunit to rapidly activate multiple downstream molecules, and the array of protein targets that can be affected by G proteins, it is not surprising that the consequences of GPCR activation within a cell are rapid and wide reaching. Moreover, because of the

many downstream consequences of G-protein signaling cascades, GPCRs have become a favorite target for drug development.

3.1.2 Small G Proteins

In addition to the heterotrimeric G proteins, there is also a superfamily of monomeric GTP binding proteins. The molecular weight of these small G proteins ranges from 20 to 40 kDa and they are subdivided into five families: Ras, Rho, Rab, Sar1/ADP ribosylation factors (ARF), and Ran. There is 30–50% homology across families and all members possess a G domain. The G domain consists of a combination of α helices and β sheets and is characterized by highly conserved loop regions that are responsible for guanine nucleotide recognition [10]. Small G proteins are activated by guanine nucleotide exchange factors (GEFs, discussed in greater detail below) to exchange GDP for GTP. Binding of GTP stabilizes the switch regions, which are critical for recognition and activation of downstream effectors. Following effector activation, the very low intrinsic GTPase activity of the small G protein is enhanced by accessory proteins and GTP is hydrolyzed to GDP, resulting in a loss of activity and a return to the GDP-bound inactive state.

The downstream effector targets of small G proteins are quite different across the superfamily. The Ras family is best known for activation of mitogen-activated protein kinase (MAPK) and its transcription factors. This pathway is initiated following ligand stimulation of receptor protein tyrosine kinases; Ras is directly activated by the GEF son-of-sevenless (Sos), and the downstream signal proceeds through the Raf/MAPK/ERK (extracellular signal-related protein kinase) cascade. Recently, the Ras family has been shown to activate other effectors as well, including p120GAP and phosphatidylinositol 3-kinase. All of these effector proteins have roles in the regulation of cell growth, morphology, and apoptosis [11]. The Rho family of small G proteins are activated by interaction with GEFs, including Dbl and Vav, and are responsible for control of the actin cytoskeleton. The members of the Rho family accomplish this task by stimulating a variety of effectors, including the well-described c-Jun N-terminal kinase (JNK) pathway and activation of p160MAPK [12]. The ARF family proteins are best known as ribosylation factors and assist in the cholera toxin-mediated adenosine diphosphate (ADP) ribosylation of the heterotrimeric G_s protein. There are many different GEFs for the ARF family, including ARF nucleotide binding site opener (ARNO) and the cytohesins [13]. The fourth family of small G proteins, the Rab proteins, are localized to distinct cellular compartments and regulate secretory and endocytic pathways. Rabs facilitate the formation of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complexes. For this purpose, the Rab proteins must be held in the GTP-bound form for prolonged periods; this task is accomplished by the Rab binding proteins, including Rabphilin and Rabaptin [14]. The final family, the Ran G proteins, are activated by GEFs such as RCC1 that are localized in or near the nucleus. This positioning of Ran and its GEFs is critical for the consequence of Ran activation—trafficking of protein and RNA in and out of the nucleus [15].

In addition to the pathways detailed here for activation of small G proteins, there is also crosstalk between these protein pathways and the signaling cascades of heterotrimeric G proteins, including downstream of G_i and G_{13} activation. Following ligand-stimulated activation and separation of the G_i heterotrimer, the $G\beta\gamma$

dimer can stimulate effector proteins. It has been demonstrated that $G\beta\gamma$ released from G_i can activate Src, which is upstream of Ras in the MAPK signaling pathway [16]. Among its other effector proteins, $G\alpha_{13}$ can bind to and activate p115RhoGEF, which directly activates Rho and its downstream signaling cascade [17]. For a few small G proteins, direct interaction with the intracellular face of a GPCR has been demonstrated, including the interaction of ARF with the M3 muscarinic receptor [18, 19]. It is not presently known if the ultimate consequence of this mechanism of small G protein activation is identical to activation through “traditional” GEFs, or if this GPCR–monomeric G protein interaction may be part of a distinct and unique cellular signaling pathway.

3.1.3 Regulation of G-Protein Cycle

For both heterotrimeric and small G proteins, regulation of GDP–GTP exchange and GTP hydrolysis is critical in controlling the initiation and duration of the G-protein signal and, consequently, the cellular response to that signal. As such, tight control of the G-protein activation cycle occurs through an array of interacting proteins. While in the GDP-bound, inactive state, small G proteins interact with accessory proteins termed GDP dissociation inhibitors (GDIs). To date, GDIs have been described for Rho and Rab. In addition to stabilizing the small G protein in its inactive, GDP-bound form, GDIs also extract GDP-bound small G proteins from the membrane and partition them in the cytosol [10].

To initiate G-protein activity, GDP must be replaced by GTP. The GDP–GTP exchange process is rate limiting in the G protein cycle and must be catalyzed for efficiency; this exchange occurs with the aid of GEFs. For heterotrimeric G proteins, the ligand-bound GPCR acts as a GEF, facilitating a conformational change in the G protein that favors exchange of GDP for GTP. For the small G proteins many distinct GEF proteins exist to accomplish this task; several of these GEFs were discussed in the context of small-G-protein activation. Similar to GPCR activation of heterotrimeric G proteins, GEFs for small G proteins are typically activated by an extracellular signal, often transduced through a receptor tyrosine kinase. GEFs are necessary to expedite the otherwise inefficient guanine nucleotide exchange process. More recently, another class of proteins termed GDP dissociation stimulators (GDSs) have been identified. The consequence of small G proteins interacting with GDSs is quite similar to the effect of GEFs, but GDS proteins have been demonstrated to lack the substrate specificity that GEFs display [20].

Once GTP is bound, the G protein is activated and may stimulate multiple effectors and/or each effector protein multiple times. While this rapid and prolific propagation of the signal is necessary, it must not remain unchecked. It has already been mentioned that small G proteins and $G\alpha$ subunits contain intrinsic GTPase activity. However, this low level of activity requires stimulation in order to be effective in signal termination. A variety of GTPase-activating proteins (GAPs) increase the rate of hydrolysis of GTP to GDP. For the heterotrimeric G proteins, many of the downstream effector targets of $G\alpha$ actually serve as GAPs, including adenylyl cyclase [21] and $PLC\beta$ [22]. In addition to these GAPs, recently identified regulators of G-protein signaling, or RGS proteins, also expedite the hydrolysis of GTP to GDP. These proteins are classified into six subfamilies based upon alignment

of their RGS domains and similarities in sequence/structure of flanking regions. Unlike the GAPs for small G proteins, the heterotrimeric RGS G proteins do not actively hydrolyze the bound GTP. Rather, they stabilize the $G\alpha$ subunit in a conformation that allows most efficient activity of the endogenous $G\alpha$ GTPase activity [23]. There is evidence from RNAi studies that RGS proteins can work in a receptor-specific manner, targeting $G\alpha$ subunits downstream of activated M3 or AT1a receptors [24, 25]. These RGS proteins may be activated by directly interacting with the specific GPCR to target $G\alpha$. In contrast to $G\alpha$ subunits, small G proteins have extremely low levels of endogenous GTPase activity and lack an Arg residue that is critical for GTP hydrolysis. Because of this, small G proteins require specialized GAPs that actively contribute to hydrolysis; proteins that can serve this function include the Ras GAP p120 and the Rho GAP p190 [11].

3.1.4 Alternate Roles for RGS Proteins

The role for RGS proteins as signal terminators has recently been expanded to include an ability to function as signal modulators and signal integrators. It has been determined that overexpressed RGS4 can effectively block/terminate G_q - and $G_{i/o}$ -mediated signaling cascades. However, when low levels of RGS4 are expressed in cells, the G_q -directed Ca^{2+} signal is not blocked, but instead a rhythmic, oscillatory Ca^{2+} response is observed [26]. This finding suggests that the complete blockade of G-protein-mediated response occurring when RGS proteins are overexpressed may be, in part, an artifact of overexpression. Physiological levels of RGS proteins are quite low in comparison, and the RGS proteins likely serve multiple functions in vivo, including but not limited to signal termination. Furthermore, while the mechanism governing this novel RGS function has not been determined, it is quite possible that the RGS proteins are acting as scaffolding proteins at the plasma membrane, assembling G proteins and their related signaling machinery into a complex, thereby enabling alternate and/or modified cell signals [26, 27].

The fact that many RGS proteins have numerous protein–protein interaction motifs, including PDZ binding motifs, pleckstrin homology, GGL ($G\gamma$ -like) and PDZ domains, provides further support to the hypothesis that RGS proteins can bring together multiple proteins and potentially act as scaffolds. One example of a direct protein–protein interaction involving RGS has been demonstrated between the R7 subfamily of RGS proteins and the $G\beta 5$ subunit [25–29]. In this dimer, the RGS protein substitutes for $G\gamma$ via its GGL interaction domain. The membrane-bound fraction of this dimer may actually associate with $G\alpha$ subunits, substituting for $G\beta\gamma$ and coupling inactive $G\alpha$ –GDP to GPCRs [25, 28], while the cytosolic fraction may merely serve to prevent recoupling with $G\alpha$, or possibly even participate in another signaling cascade through its documented interaction with the scaffolding protein 14-3-3 [26, 30].

RGS proteins can also provide links between heterotrimeric and monomeric G-protein signaling cascades. The RGS protein p115-RhoGEF is activated downstream of $G\alpha_{13}$ –GTP; p115-RhoGEF then activates the Rho signaling cascade. Interestingly, not only is p115 a GEF for Rho, it also possesses potent GAP activity for $G\alpha_{12/13}$ subunits; thus it is able to negatively regulate the very protein that caused its activation. In addition to p115RhoGEF, G-protein receptor kinases (GRKs) are RGS proteins that also have multiple interacting partners and well-described cellular

roles apart from their RGS/GTPase function. GRKs are perhaps best known for their role in receptor phosphorylation/desensitization; however, they also possess a low level of RGS activity, further contributing to their ability to turn off specific G-protein signals.

3.1.5 G Proteins and Disease

While the downstream signals from G proteins are critical for proper cell regulation, response, and survival, these signals can also be detrimental for the cell if left unregulated. In a variety of tumors, this is precisely what occurs. Small G proteins have many well-documented oncogenic mutations, including mutations that prevent GTP hydrolysis, leaving the G protein permanently in the “on” state and mutations that enhance GDP–GTP exchange, promoting an active state of the G protein [31, 32]. This constant signal results in unchecked cell growth and abnormal cell cycle progression and migration. Not only do these oncogenic mutations cause tumor initiation, but they may also contribute to tumor invasion and metastasis [33].

Mutations within the genes encoding $G\alpha$ are rare and lead to distinct disorders. A mutation resulting in a gain-of-function (or permanent on state) in the $G\alpha_s$ protein leads to the endocrine disorder McCune–Albright syndrome [34, 35]. Loss-of-function mutations in $G\alpha_s$ are also disease-causing, as evidenced by the congenital syndrome Albright hereditary osteodystrophy. This disorder is caused by a partial loss of $G\alpha_s$ function, termed haploinsufficiency [34, 35]. G proteins can also be targeted for modification by bacterial toxins; this is the mechanism of action of both cholera and pertussis toxin. Cholera toxin inhibits hydrolysis of GTP from $G\alpha_s$ –GTP, thus leaving the G protein in an active state, while pertussis toxin ribosylates the $G_{i/o}$ heterotrimer, preventing activation of the G protein. The importance of G proteins in cellular communication and regulation is underscored by the deleterious effects caused by mutations and posttranslational modifications of these proteins.

In addition to the diseases described here, it is quite likely that dysfunction of monomeric and heterotrimeric G proteins contributes to many polygenic disorders with undefined etiology. While much is already known about G proteins, there are still obvious gaps in our knowledge, and new discoveries in the field alter how we perceive cellular signaling mechanisms and interactions. By further expanding our knowledge of G-protein structure, function, and activation/deactivation, we can move closer toward understanding the importance of these proteins not only in normal cellular function but also in diseased conditions. This knowledge could enable the development of therapeutics that target G proteins as well as their activators and effectors, yielding novel disease treatments.

3.2 GPCR–G PROTEIN MODULATION OF ION CHANNELS

Communication between neurons across synapses requires the release of presynaptic neurotransmitters and postsynaptic receptor activation. This synaptic transmission in the nervous system has long been known to be mediated by ion channels, which, when activated by a transmitter, allow charged ions to pass through the opened channels resulting in a change of potential across the postsynaptic cell membrane. GPCRs, which are directly coupled not to ion channels but to G proteins, as

discussed in earlier sections, were once generally thought not to play a predominant role in synaptic fast (ionotropic) transmission. However, evidence now supports an indispensable role for GPCRs in controlling the efficacy of this transmission by modulating the function of ligand-gated ion channels. Furthermore, GPCRs also mediate slow synaptic transmission via regulation of voltage-gated calcium and potassium ion channels as well as through their more well known role of generating second messengers.

GPCRs are able to influence ion channels through two distinct mechanisms. One involves the modulation of ion channels by diffusible second messengers either through G proteins or through downstream signaling molecules such as cAMP, PKA, or PKC. The second is through direct protein–protein interactions between ion channels and GPCRs. The latter mechanism is less understood even though it allows for a more specific form of regulation. Both G-protein- and second-messenger-mediated regulation of ion channels as well as direct GPCR-ion channel interactions will be reviewed.

3.2.1 $G\beta\gamma$ Signaling to Ion Channels

Modulation of ion channels by diffusible G proteins can be both indirect (via stimulation of second messengers) and direct (via physical interactions between G-protein subunits and the channel protein). The most intensely studied directly interacting G protein with ion channels is the $G\beta\gamma$ dimer. As discussed above, after activation of the GPCR, conformational changes in the $G\alpha\beta\gamma$ heterotrimer facilitate the exchange of GDP for GTP on the α subunit and the ensuing dissociation of $G\beta\gamma$ from $G\alpha$. The GTPase activity of the $G\alpha$ subunit reverses this process [36]. It is during this stimulation and subsequent dissociation that $G\beta\gamma$ is free to activate a large number of effectors. The most intensely studied and best understood example of ion channel regulation by $G\beta\gamma$ is the G-protein-activated inwardly rectifying K^+ (GIRK) channel [37, 38]. GIRK channels are expressed in the heart, central and peripheral nervous systems, and endocrine system where they control postsynaptic inhibitory signaling and hormone secretion [39]. In the central nervous system (CNS), activation of GIRK channels mediates the inhibitory actions of γ -aminobutyric acid (GABA), acetylcholine, adenosine, somatostatin, and opioids. The clinical importance for GIRK channels in the CNS has been elucidated via use of the compound flupirtine, a centrally acting nonopioid analgesic that activates pertussis toxin-sensitive GIRK channels, leading to an indirect inhibition of *N*-methyl-D-aspartate (NMDA) receptor activity [40]. Studies with this compound have shown that it protects neurons from the toxic effects of prion proteins, amyloid- β and glutamate. These data suggest that activation of GIRK channels may be of therapeutic value and a target for the treatment of neurodegenerative disorders [41].

The regulation of GIRK channels proceeds as follows: the $G\beta\gamma$ protein directly binds to and activates the channel [42]. This creates a linking mechanism between GPCR activation and the regulation of ion channel function. The precise mechanism of how a diffusible protein ($G\beta\gamma$) is able to activate the GIRK is beginning to be unraveled. Specific amino acids in the channel pore that when mutated render the channel both constitutively active and insensitive to $G\beta\gamma$ activation have suggested that $G\beta\gamma$ allosterically regulates the gating apparatus of the channel pore. $G\beta\gamma$ binds to the pore and likely causes a conformational rearrangement of the transmembrane

segment of the GIRK, capable of widening or narrowing the pore. The rearrangement is such that it results in GIRK channel activation [39]. Through this mechanism, GPCR activation of G_i proteins elicits profound effects on GIRK channel function (Fig. 3.2a) [43].

Importantly, it has been elucidated that GIRK channels are regulated by multiple mechanisms by GPCRs. In systems that contain both G_i -coupled GPCRs and G_q -coupled receptors, coactivation of the GPCRs results in competing effects on the GIRK. Simultaneous stimulation of both G_i - and G_q -coupled GPCRs activates GIRK initially through $G\beta\gamma$ as discussed above, but stimulation of PLC by $G\alpha_q$ reverses this activation. It does this by decreasing phosphatidylinositol-4,5-bisphosphate (PIP₂) and increasing diacylglycerol (DAG). DAG in turn recruits PKC, resulting in phosphorylation of the GIRK and subsequent decreased interaction between GIRK and PIP₂, resulting in channel inhibition (Fig. 3.2b). [43]. Therefore, different GPCRs are capable of regulating the same ion channels via different mechanisms, resulting in opposite functional outcomes.

GIRK channels are not the only ion channels modulated by diffusible $G\beta\gamma$ proteins generated through activation of GPCRs. Whereas GIRK channels are activated by GPCR-stimulated liberation of $G\beta\gamma$, voltage-gated Ca^{2+} channels (VGCCs) are inhibited. GPCR activation was first found to reduce action potential duration in dorsal root ganglion neurons in the 1970s. This observation was subsequently confirmed to be due to inhibition of VGCCs, and this effect has since

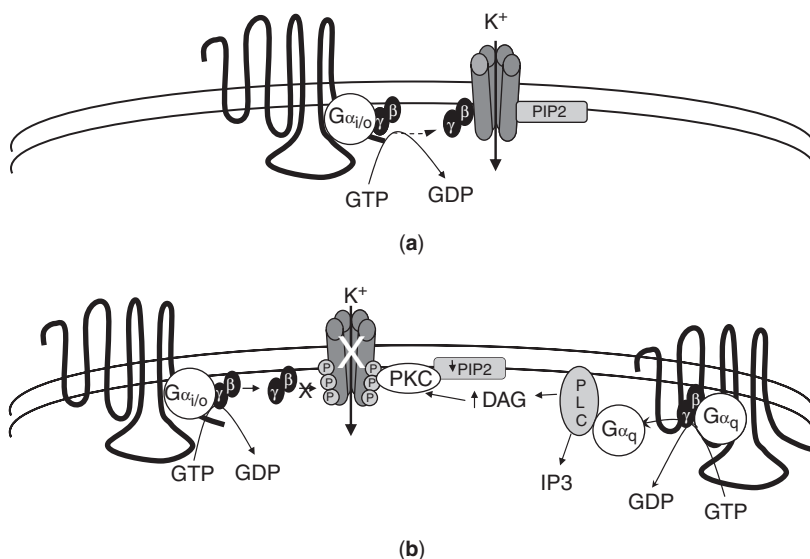


Figure 3.2 Modulation of GIRK channels by GPCRs. (a) Activation of GIRK channels by GPCR stimulation. Agonist binding to G_i -protein-coupled GPCR results in the liberation of $G\beta\gamma$, which in turn binds to the GIRK channel and activates it in the presence of PIP₂. (b) Inhibition of GIRK channels by GPCRs. Simultaneous stimulation of both G_i - and G_q -coupled GPCRs activates GIRK initially, but G_q stimulation of PLC decreases PIP₂ and increases diacylglycerol (DAG), which recruits PKC. Phosphorylation of GIRK subunits results in decreased interaction with $G\beta\gamma$ and PIP₂ and channel inhibition. Adapted from [43] with permission.

been observed in many types of neurons (for review, see [44]. VGCCs play a major role in the functioning of nearly all neurons and other excitable cells being crucial for depolarization and transmitter release [44]. Studies have revealed that $G\beta\gamma$ binding to VGCCs results in inhibition of the calcium current manifested by slowing of the current activation kinetics. GPCRs typically involved in this type of modulation include α_2 adrenoceptors, opioid receptors, and GABA-B receptors [44]. The mechanism behind this inhibition occurs through a series of convergent modifications in the biophysical properties of the channel. This likely involves the reluctance of VGCCs to open when they are bound to G proteins and may require that the $G\beta\gamma$ be dissociated before the channel is able to open normally (for review, see [45]). A simple model would be that on any given cell only the non- $G\beta\gamma$ -bound channels open while the bound ones do not, leading to an overall decrease in VGCC gating. This inhibition is fast and relieved by depolarization, likely through the dissociation of the G protein from the channel allowing for opening. In this way, GPCRs are able to regulate neurotransmission through the diffusible G protein $G\beta\gamma$ acting on VGCCs.

While extensive data have now demonstrated an indisputable role for $G\beta\gamma$ regulation of GIRK and VGCC, several other ion channels also have the potential to be regulated via this mechanism, including voltage-dependent K^+ channels [46], and adenosine triphosphate (ATP) sensitive K^+ channels [47]. Other, yet to be determined ion channels may show similar forms of regulation, indicating a more widespread effect of $G\beta\gamma$. Regardless of the channel affected, signaling through this mechanism is likely regulated in dynamic multi molecular complexes depending on cell type and localization of receptors in similar compartments allowing for selective signaling [48].

In addition to $G\beta\gamma$ binding directly to ion channels and modulating their opening, the heterodimer is also known to elicit responses on downstream signaling molecules such as kinases and small G proteins [2]. These downstream signaling molecules also have a vast potential to regulate ion channels, particularly through phosphorylation events. Additional downstream GPCR signaling molecule effects on ion channels will be discussed below.

3.2.2 Other Downstream Signaling Molecules That Modulate Ion Channels

As will be discussed in detail in subsequent sections, when GPCRs are activated, in addition to allowing for the dissociation of diffusible G proteins, they are further directly responsible for activating downstream signaling complexes such as cAMP, PKA, and PKC. Interestingly, these downstream signaling events are also capable of regulating ion channels in a variety of methods. These mechanisms increase the complexity of GPCR control on ion channels, allowing for more specificity of the interaction in controlling different receptors. This also allows for additional control of the ion channel by other factors that regulate these downstream signaling pathways. Perhaps surprisingly, multiple ion channels are regulated by both direct interactions with G proteins and indirectly via downstream signaling pathways, greatly increasing the crosstalk between GPCRs and ion channels.

Phosphorylation is a common mechanism for the modulation of ion channels, and this event is directed by kinases, some of which are stimulated via GPCR activation. Both voltage-gated and ligand-gated ion channels are regulated via phosphorylation controlling such events as receptor desensitization and activation [49]. VGCCs are

one example of a voltage-gated ion channel that is regulated by both mechanisms: $G\beta\gamma$ proteins and second-messenger protein kinases [50]. VGCCs are a substrate for phosphorylation by cAMP-dependent protein kinase [51]. In addition, modulation of calcium currents by PKC can occur through the initial activation of G_i or G_q resulting in the activation of PLC. In neurons, β -adrenergic and D_1/D_5 dopamine receptors can modulate some types of calcium channels through cAMP-dependent mechanisms. Some types of neuronal VGCCs are enhanced by activation of PKC, whereas other reports show that some types of VGCCs are inhibited by PKC. In addition, it has been shown that integration of GPCR signaling can take place by regulating the interaction of the VGCCs with different cellular proteins [50]. Taken together it is clear that multiple GPCR-mediated pathways converge to modulate ion channels in a variety of diffusible methods, including downstream regulators. The complex timing of events is controlled by interactions between components of multiple signaling pathways. These multiple mechanisms allow for the integration of a complex system where GPCR activation can modulate ion channels to control cellular transmission.

VGCCs represent just a single example of the many effects that downstream second-messenger signaling via kinases plays in phosphorylation and subsequent regulation of a number of ion channels. For example, stimulation of PKA by the D_1 dopamine receptor is able to affect voltage-gated K^+ channels, Na^+ channels, Ca^{2+} channels, NMDA receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole propinoic acid (AMPA) receptors, and GABA receptors. To further complicate this regulation, PKC stimulation by this same GPCR (D_1 dopamine receptor) is capable of modifying some of the same channels, including Na^+ channels and Ca^{2+} channels [52]. Overall, it is easy to appreciate the complex regulation of ion channels by GPCR-stimulated protein kinases resulting in ion channel phosphorylation. With so many converging downstream regulators how can an individual cell determine which one is most influential? This is most likely a case of subcellular localization of the GPCRs and the affected ion channel; for example, when they are in the same cell type and in close proximity, they can affect each other. What actually occurs in vivo (the cumulative effect of multiple cellular systems eliciting control over the ion channel regulation) continues to be investigated.

3.2.3 Modulation of Ion Channels by Direct Protein–Protein Interactions with GPCRs

While we have discussed several means by which stimulation of GPCRs regulate the function and expression of ion channels, all of these were via indirect diffusible second-messenger-linked mechanisms. However, it is also becoming clear that GPCRs are able to regulate some ion channels through direct protein–protein interactions. While it is known that neurons contain many different types of GPCRs, they activate only a limited number of G-protein complexes and second-messenger pathways. Therefore, a potential challenge for the cellular regulation of ion channels by second-messenger molecules is regulating the high degree of selectivity and specificity that occurs inside any given cell. Some of these specificity issues may be addressed by direct protein–protein interactions between the GPCR and an ion channel. The direct protein–protein interactions appear to be functionally reciprocal in most cases,

allowing for the receptors to regulate each other. The majority of data examining this direct interaction involves ligand-gated ion channels interacting with GPCRs.

A growing body of evidence suggests that direct interaction of GPCRs and ligand-gated ion channels represents a mechanism by which two types of receptors work in concert to regulate synaptic transmission. One example of direct interactions between an ion channel and a GPCR is dopamine receptors interacting with GABA_A channels [53]. In this case the receptors comodulate each other, where activation of the GPCR (in this case D₅ dopamine receptor) inhibits GABA-mediated synaptic transmission and activation of the GABA receptor inhibits D₅ receptor-mediated cAMP accumulation. This reciprocal regulation is not via diffusible second messengers but by direct protein-protein interactions. The mechanism for this is likely cointernalization, where activation of one receptor initiates ligand-induced internalization and, because the two receptors are linked, internalizes the other receptor with it. While this mechanism is not entirely clear, it does highlight a mechanism by which GPCRs can modulate ion channels in a way that does not require diffusible second messengers.

The interaction of the D₁ dopamine receptor with glutamate receptors represents another complex regulatory paradigm where the ion channel is regulated via both diffusible second messengers and direct protein-protein interactions. D₁-like dopamine receptors enhance NMDA receptor-mediated responses by signal integration of cAMP response element binding (CREB) protein and PKA-dependent phosphorylation (for review, see [52]). Furthermore, D₁ receptor stimulation also enhances trafficking of the NMDA receptors and the GluR1 subunit of AMPA receptors [54, 55]. The GluR1 trafficking is mimicked by forskolin-induced activation of adenylyl cyclase and the NMDA receptor effect requires the Src family protein tyrosine kinases Fyn (for review, see [52]). Interestingly, the NMDA receptor is also regulated by direct protein-protein interactions with the C-terminus of the D₁ receptor, allowing the receptors to reciprocally regulate each other.

The direct protein-protein interactions between the D₁ receptor and the NMDA receptor is complex and results in multiple regulatory effects between the two receptors [56, 57]. The two receptors directly interact through not one but two distinct mechanisms—one with the C-terminus of the D₁ receptor and the NR1 subunit of the NMDA receptor and another between the C-terminus of the D₁ receptor and the NR2A subunit of the NMDA receptor [57]. Interestingly, it appears that these two distinct interaction sites mediate disparate functional consequences for GPCR-ion channel interactions. For the interaction with the NR2A subunit when the dopamine receptor is stimulated, the interaction results in inhibition of NMDA receptor-mediated ion currents. This effect is not due to any of the major signaling cascades known to be downstream of the D₁ dopamine receptor, since interference with PKA, PKC, and G proteins has no effect on this inhibition. Therefore, it is due to direct protein-protein-mediated changes in the receptor. In contrast, the other interaction (the one with the NR1 subunit) appears to be involved with another D₁-mediated effect, the suppression of NMDA receptor-mediated cell death [57]. This effect is not mediated by the inhibition of NMDA currents like that seen for the other interaction. Similar to the effects of D₁ stimulation on NMDA currents, this blockade of NMDA-mediated cell death does not involve G proteins. The precise mechanism of how these protein-protein interactions result in these functional effects remains unclear but may involve internalization of the ion channel following activation of the GPCR.

3.3 SIGNALING THROUGH CYCLIC NUCLEOTIDE SECOND MESSENGERS

GPCRs detect the presence of neurotransmitters, hormones, and other cellular activators by physically interacting with these signal-initiating molecules—the first messengers—and communicating their presence by activating intracellular heterotrimeric G proteins. Activated G proteins, in turn, modulate cellular effectors such as adenylyl cyclase (AC) that synthesize and regulate the amount of second messenger present inside the cell. The cyclic nucleotides adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) constitute the second messengers that are synthesized by the cellular effectors AC and GC. Once synthesized, second messengers go on to modulate a variety of cellular processes by activating protein kinases, gating ion channels, and modulating transcription factors to attune the cell's physiology in response to exposure to the first messengers—the culmination of the signal transduction cascade.

3.3.1 Adenylyl Cyclase

Adenylyl cyclase was the first cellular effector target identified for activated G protein. This observation led to the original hypothesis made by Sutherland and Rall that a single polypeptide could both recognize hormonal signals and synthesize adenosine-3',5'-monophosphate, that is, cAMP. We now know that cAMP production requires at least three proteins: (1) a membrane-bound receptor that recognizes and binds cognate agonist (first messenger) to activate associated (2) heterotrimeric G proteins that in turn bind and activate the (3) membrane-bound AC enzyme that catalyzes the transformation of ATP into cAMP. Figure 3.3 illustrates the prototypical hormone-sensitive AC signal transduction system.

Heterotrimeric G-protein regulation of AC activity is mediated by both the α and $\beta\gamma$ subunits. The α -subunit regulation of AC activity can be either stimulatory ($G\alpha_s$) or inhibitory ($G\alpha_i$) as is the $\beta\gamma$ -subunit regulation of AC activity. Table 3.2 summarizes the regulation of the various isoforms of AC by G protein. Hormonal activation of ACs is mediated predominantly by $G\alpha_s$ -coupled GPCRs, with $G\alpha_s$ being the most widely distributed activator of membrane-bound ACs in mammals [58]. Most of the focus of G-protein regulation of ACs was placed on the α subunits; however, $\beta\gamma$ dimers represent some of the most effective negative regulators of AC1 and AC8 [59]. Interestingly, the AC isoforms (AC2, AC4, and AC7) that are activated by $\beta\gamma$ dimers are not directly regulated by the α subunits of G_i proteins [58, 60]; thus, the modulation of AC activity by heterotrimeric G proteins is a complex process that exhibits AC isoform-specific regulation, providing a platform for crosstalk among different signal transduction systems.

Calcium ions represent an alternative modulator of AC activity that can also be either stimulatory or inhibitory. G proteins that do not directly bind AC can still modulate AC activity by gating ion channels that increase intracellular calcium ion concentration. AC1, AC3, and AC8 activity can be stimulated by Ca^{2+} in complex with the protein calmodulin [61, 62]. The mechanism via which Ca^{2+} /calmodulin (Ca^{2+} /CaM) activates AC depends on the particular isotype of AC. For example, activation of serotonin 5-HT_{7A} receptor stimulates AC8 activity, but this activation is mediated not by $G\alpha_s$ binding but by interaction of AC8 with Ca^{2+} /CaM [63]; thus,

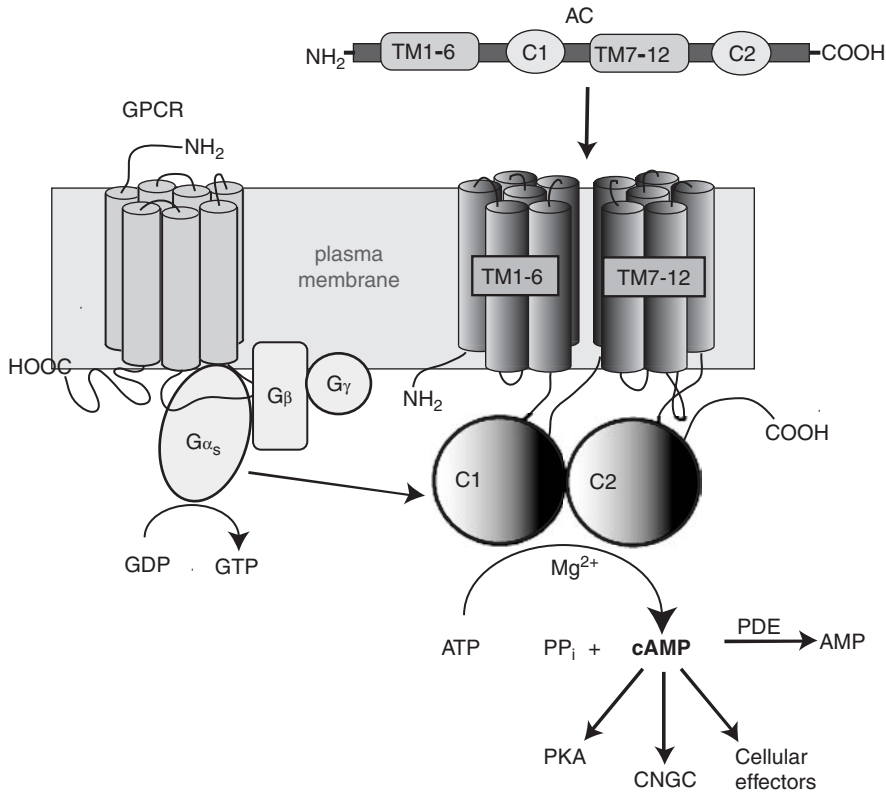


Figure 3.3 Prototypical activation of adenylyl cyclase (AC). Hormone binds to a G α_s linked GPCR inducing the exchange of GDP for GTP on the G α subunit of associated heterotrimeric G protein, resulting in activation of G protein. Activated G α_s dissociates from the $\beta\gamma$ dimer and binds to and activates the catalytic region (C1–C2) of AC. AC is a single polypeptide that exhibits a bipartite arrangement of a six-transmembrane (TM) spanning region followed by an intracellular catalytic domain (C) configured into an intramolecular dimer. The linear domain arrangement of AC is depicted at the top as a horizontal bar diagram. The overall structure of AC resembles a membrane channel with both amino and carboxyl termini located within the cell as depicted by the bipartite barrel structure above. Activated AC synthesizes cAMP second messenger from cytosolic ATP, releasing pyrophosphate (PP_i). cAMP can go on to modulate the activity of a variety of cellular effectors, including cAMP-dependent PKA and cyclic nucleotide-gated channels (CNGCs), or be degraded by phosphodiesterase (PDE) resulting in attenuation cAMP signaling.

AC8 acts as a calcium ion detector with half-maximal stimulation achieved at 800 nM free Ca²⁺ [64]. AC1 is more sensitive to Ca²⁺ than AC8, achieving half-maximal activity at 150 nM of free Ca²⁺ [65]. Moreover, AC1 is also activated by G α_s . Activated G α_s and Ca²⁺/CaM synergistically stimulate AC1, causing AC1 to act as a coincidence detector to integrate increases in intracellular Ca²⁺ and activation of G α_s -GPCRs to enhance cAMP production [64]. Inhibition of AC, specifically AC5 and AC6, by Ca²⁺ does not require association with calmodulin and is mediated by both a low-affinity state that occurs in all tissues and a high-affinity state that occurs in select tissues, reviewed in [66]. Interestingly, calcium regulation of ACs is

dependent upon the method of how intracellular Ca^{2+} concentration is increased. An increase in intracellular calcium achieved by the opening of intracellular stores or by ionophore-mediated or arachidonic acid-mediated entry of external calcium through the plasma membrane does not alter AC activity, reviewed in [66]. Modulation of AC activity, especially the inhibition of AC5 and AC6, is linked to capacitative calcium entry (CCE)—the entry of calcium through the plasma membrane that is initiated upon depletion of intracellular stores, reviewed in [66, 67]. Transient receptor potential (TRP) channels are thought to be the plasma membrane channels that mediate the effective CCE current (reviewed in [68]).

Additional regulators of ACs that can be stimulatory or inhibitory include the protein phosphatase calcineurin and the following protein kinases: cAMP-dependent protein kinase (PKA, discussed below), PKC, and Ca^{2+} /CaM kinases (reviewed in [60, 69]). Several examples exist for inhibition of AC activity via direct phosphorylation by protein kinases. AC1 has been shown to be phosphorylated by CaM kinase IV *in vivo* and this phosphorylation results in a decrease in AC1 catalytic activity [70]. AC5 has been shown to be phosphorylated by PKA and this phosphorylation results in a decrease in the rate of AC5 generation of cAMP [71]. Interestingly, AC5 has also been shown to be phosphorylated by PKC α and PKC ζ at sites distinct from the PKA phosphorylation site, resulting in a 10- to 20- fold increase in AC5 catalytic activity [72].

Several chemical compounds have also been used to alter AC activity to examine the behavior of the enzyme under a variety of experimental conditions. All mammalian ACs are activated directly by the membrane-permeable diterpene forskolin, except for AC9 and the soluble form of AC (sAC). Use of forskolin alleviates the need for GPCR–G protein or calcium activation of AC to potentiate cAMP production. Conversely, P-site inhibitors have been used to inhibit AC activity in a manner that does not compete with ATP binding to the enzyme, reviewed in [58]. P-site inhibitors are mono- or polyphosphate structural analogs of cAMP. They inhibit AC activity by stabilizing the quasi-product-bound state. The capacity of P-site inhibitors to attenuate AC activity is dramatically increased by the presence of pyrophosphate.

Molecular cloning and expression experiments investigating sAC revealed that this isotype was insensitive to both G-protein and forskolin activation but highly sensitive to stimulation by bicarbonate ion [HCO_3^-] [73]. Several metabolic processes result in changes in the CO_2 content of cells. These changes are buffered when HCO_3^- , CO_2 , and H^+ reach equilibrium within the cell; hence, changes in energy usage as well as cellular pH would shift the concentration of reactants according to the buffering capacity of the reaction. Thus any changes that would increase HCO_3^- concentration would stimulate sAC to produce cAMP, making sAC a pH and CO_2 sensor within a cell [74]. Identification and characterization of sAC have forced a reexamination of how cAMP signals are propagated within the cell beyond activation of GPCRs at the plasma membrane [58].

Molecular cloning has identified a total of 10 isotypes of AC: 9 membrane-bound isoforms [58, 69] and sAC isoform [73]. The genes that correspond to the AC isoforms are distributed throughout the genome on multiple chromosomes, with the exception of AC7 [75] and AC9 [76], which are located on human chromosome 16, reviewed in [58]. Mammalian ACs are expressed in every tissue at very low levels; however, some isoforms are more ubiquitously expressed than others. AC1 and AC8

are expressed exclusively in neurons [61, 77] and are localized predominantly to regions in the brain associated with learning and memory. Table 3.2 summarizes tissue expression patterns of each AC isoform.

Combination of the molecular cloning sequence data with the information available on the modulation of the various AC isoforms enables the membrane-bound ACs to be grouped into three distinct families as follows: (1) Ca^{2+} /CaM-stimulated family that consists of AC1, AC3, and AC8; (2) the Ca^{2+} -insensitive, PKC- and $\text{G}\beta\gamma$ -stimulated family comprised of AC2, AC4, and AC7; and (3) the high-affinity Ca^{2+} - and $\text{G}\alpha_i$ -inhibited family comprised of AC5 and AC6 [58]. AC9 is the most divergent of the mammalian membrane ACs [78]. It is unique in that it is the only membrane-bound AC that is virtually insensitive to activation by forskolin. AC9 has been shown to be stimulated by $\text{G}\alpha_s$ [78, 79], and this stimulation is attenuated by activation of a novel PKC in HEK293 cells [79]. AC9 was found to be unresponsive to $\text{G}\beta\gamma$, Ca^{2+} , or Ca^{2+} /CaM treatment [78].

3.3.2 Cellular Targets of cAMP

Activation of AC by any of the methods discussed above results in the conversion of ATP into the second messenger cAMP and pyrophosphate. Conversely, cAMP can be hydrolyzed by enzymes known as phosphodiesterases (PDEs) to produce AMP and, hence, turn off cAMP signaling (Fig. 3.3). cAMP signaling is predominantly carried out by the binding of cAMP to and activation of the cAMP-dependent protein kinase (PKA).

PKA is a serine/threonine-directed kinase that exists as a tetrameric enzyme consisting of two regulatory subunits and two catalytic subunits. There are currently three catalytic subunits ($\text{C}\alpha$, $\text{C}\beta$, and $\text{C}\gamma$) and four regulatory subunits ($\text{RI}\alpha$, $\text{RI}\beta$, $\text{RII}\alpha$, and $\text{RII}\beta$) identified for PKA [80, 81]. It is the specific homo- and/or heterodimerization of the regulatory subunits that distinguishes the two forms of PKA holoenzyme: type I (consisting of RI subunits) is predominantly cytoplasmic and more sensitive to cAMP [82] and type II (consisting of RII subunits) is associated with specific cellular structures and organelles (reviewed in [83]). Each regulatory subunit consists of an N-terminal dimerization domain, an autophosphorylation site that binds the catalytic subunits, and two cAMP binding sites [84]. The regulatory domains of PKA serve two major functions: first, as an autoinhibitory module for the catalytic subunits in the absence of cAMP and, second, as a dimerization and docking domain (D/D domain) to localize the tetrameric holoenzyme to specific subcellular locations through association with A-kinase anchoring proteins (AKAPs) (reviewed in [85]). Upon synthesis, two molecules of cAMP bind to each regulatory subunit inducing activation and dissociation of the catalytic subunits from the regulatory subunits. The activated catalytic subunits are free to phosphorylate a diverse array of cellular substrates in both the cytoplasm and the nucleus. For example, phosphorylation of the transcription factor CREB in the nucleus by the catalytic subunits of PKA regulates gene transcription at CRE (cAMP response element) sites in promoter regions [86]. PKA can also phosphorylate PDE3A and PDE4, activating the enzymes to degrade cAMP, providing a negative-feedback loop to maintain levels of the second messenger in smooth muscle cells [87].

In addition to PKA, cAMP can also bind and regulate cyclic nucleotide-gated (CNG) channels in the cilia of olfactory sensory neurons [88] and cardiac pacemaker

TABLE 3.2 Regulatory Properties of Mammalian Adenylyl Cyclases

AC Isoform ^{a,b}	Tissue Distribution	Regulation by G-Protein Subunit	Protein Kinases	Calcium Effects	Putative Function
AC1	Brain adrenal medulla	Stimulated by $G\alpha_s$, Inhibited by $G\beta\gamma$, Inhibited by $G\alpha_0$	PKC: weak stimulation; CaMKIV: inhibition	Stimulated by Ca^{2+} -CaM inhibition	Learning, memory, LTP ^c , synaptic plasticity
AC2	Brain, skeletal muscle, lung, heart	Stimulated by $G\alpha_s$, Stimulated by $G\beta\gamma$ ^d	PKC: stimulation		
AC3	Brain, olfactory epithelium	Stimulated by $G\alpha_s$	PKC: weak stimulation; CaMKII: inhibition	Stimulated by Ca^{2+} -CaM	Olfaction
AC4	Brain, heart, kidney, liver, lung, BAT, uterus	Stimulated by $G\alpha_s$, Stimulated by $G\beta\gamma$ ^d	PKC: inhibition		
AC5	Heart, brain, kidney, liver, lung, uterus, adrenal, BAT ^c	Stimulated by $G\alpha_s$, Inhibited by $G\beta\gamma$ ^e , Inhibited by $G\alpha_s$	PKA: inhibition, PKC α,ζ : stimulation	Inhibited	
AC6	Ubiquitous	Stimulated by $G\alpha_s$, Inhibited by $G\beta\gamma$ ^e , Inhibited by $G\alpha_f$	PKA: inhibition; PKC: inhibition	Inhibited	
AC7	Ubiquitous, highly expressed in brain	Stimulated by $G\alpha_s$, Stimulated by $G\beta\gamma$ ^d	PKC: stimulation		Drug dependency

AC8	Brain, lung, testis, adrenal, uterus, heart	Stimulated by $G\alpha_s$	Stimulated by Ca^{2+} -CaM	Learning memory, LTP ^c , synaptic plasticity
AC9 ^g	Brain, skeletal muscle	Stimulated by $G\alpha_s$		
sAC ^h	Highly expressed in testes	Not regulated by G -protein subunits		Sperm capacitation

Source: From *Molecular Intervention*, 2 (3), by R. K. Sunahara and R. Taussing. "Isoforms of mammalian adenylyl cyclase: multiplicities of signaling," pp168–184 (2002). Adapted with permission from ASPET.

^aAll isoforms except sAC are inhibited by P-site inhibitors.

^bForskolin stimulates human AC1-8, whereas AC9 is weakly and sAC is not stimulated by forskolin.

^cBAT, brown adipose tissue; LTP, long-term potentiation.

^d $G\beta\gamma$ stimulation of AC is conditional upon $G\alpha_s$ co-activation.

^eInhibition determined by transfection and could be an indirect $G\beta\gamma$ effect.

^fDenotes $G\alpha_i$ family member $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_z$.

^gInhibited by calcineurin.

^hStimulated by bicarbonate.

channels in the sinoatrial node [89]. Both CNG channels are regulated by direct binding of cAMP. cAMP can also bind to EPAC—a chimeric protein comprised of a cAMP binding domain fused to a GEF domain that specifically activates the small G protein Rap1 of the Ras superfamily. Binding of cAMP to this GEF activates Rap1 in a PKA-independent manner [90].

3.3.3 Guanylyl Cyclase

Guanylyl cyclases (GCs) are the cellular effectors that synthesize the second messenger cGMP from GTP, reviewed in [91]. GCs are involved in physiological processes such as vision, bone growth, and vasodilation. GCs display a dramatically different structure from that of the ACs; however, there is considerable sequence similarity in the catalytic regions of each class of cyclase that produce the cyclic second messengers [92]. Mammalian GCs exist in two forms—membrane bound and soluble—as do the ACs. In contrast to the membrane-bound ACs, the membrane-bound GCs directly bind agonist to initiate cGMP production instead of activation via an agonist–GPCR–G protein avenue. Figure 3.4 depicts the domain structure of mammalian GCs.

Soluble GC (sGC) lacks the extracellular domain (ECD), the transmembrane (TM) domain, and the kinase homology domain (KHD) present in the membrane-bound analogues. Instead, the sGC contains an N-terminal heme domain that binds nitric oxide (NO)—the uncharged free-radical compound that stimulates cGMP production in this enzyme [93]. NO is produced from L-arginine by nitric oxide synthase (NOS) [94]. sGC exists as a heterodimer, with α_1/β_1 being the most abundant species. sGC regulates smooth muscle relaxation, platelet aggregation, and synaptic transmission [91, 95]. Direct desensitization of sGC has not yet been observed; however, signaling through sGC is inhibited downstream via PDE5 and cGMP-dependent protein kinase (PKG) [95].

The membrane-bound (also referred to as particulate) GC family is composed of seven members that also exist as dimers. The atrial natriuretic peptide receptor (GC-A) was the first to be cloned and is activated by atrial or brain natriuretic peptides (ANP or BNP) leading to a net decrease in blood pressure. GC-B is very similar to GC-A but is only activated by c-type natriuretic peptide (CNP) and is mainly associated with bone growth. GC-C is activated by the three related endogenous peptides guanylin, uroguanylin, and lymphoguanylin. GC-A, GC-B, and GC-C are the only GCs where the cognate activator molecules bind to the ECD even though this domain is present in all membrane-bound GCs. Glycosylation and a deeply buried chloride ion are required for proper folding of the ECD to achieve maximal ligand efficacy (reviewed in [91]). In addition, the KHD of these three GCs is a negative regulatory domain that does not possess kinase activity yet requires the binding of ATP to achieve activation of the enzyme [96, 97]. GC-E (or retGC-1) and GC-F (or retGC-2) are retinal GCs that restore intracellular calcium in photoreceptor cells upon light activation [98]. Activation of these GCs differs from GC-A and GC-B in that the activation domain is located intracellularly and is mediated by separate proteins called GC-activating proteins (GCAPs). Ca^{2+} -bound GCAPs bind to the coiled-coil region of the GC-E/GC-F dimer to activate cGMP synthesis (see Fig. 3.4) [99]. The remaining two members of the family GC-D and GC-G are orphan

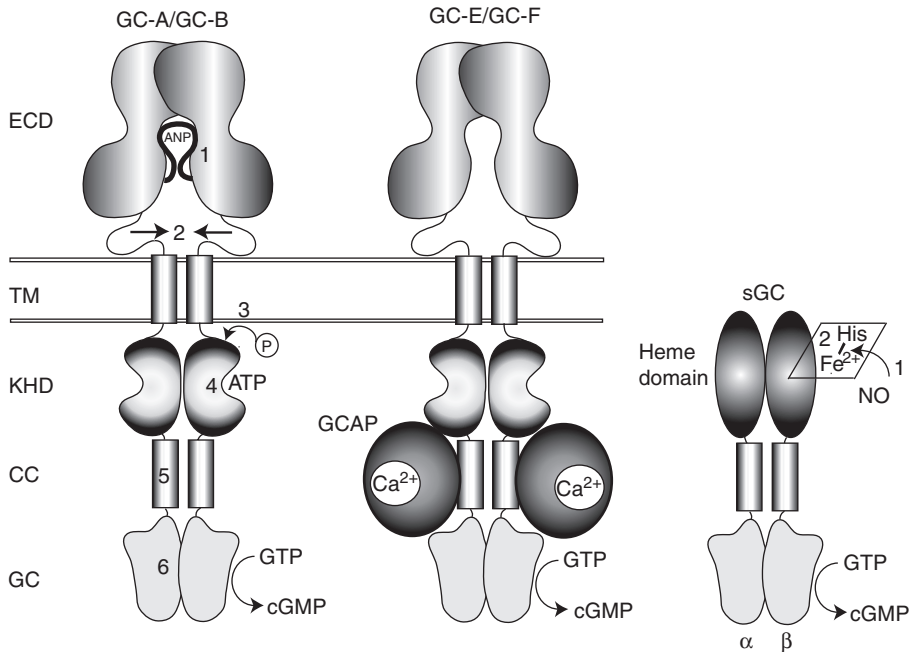


Figure 3.4 Domain structure of mammalian guanylyl cyclases (GCs). The membrane-bound GC architecture is as follows: extracellular domain (ECD), transmembrane helix (TM), kinase-homology domain (KHD), coiled-coil region (CC), and GC domain. For clarity, the GC-activating protein (GCAP) is shown with only one of its three calcium binding sites. The sites of the postulated conformational changes during GC receptor activation for both the natriuretic peptide receptor and soluble GC are numbered. For GC-A/GC-B, these conformational changes include hormone binding (1), juxtapositioning of the ECD C-termini (2), phosphorylation (3), ATP binding (4), coiled-coil adjustments (5), and conformational changes in the GC domain (6). The precise order of these conformational changes is not known. Furthermore, the activation mechanism likely involves multiple conformational changes at some of these sites to account for outside-in and inside-out signaling as part of the receptor's ability to alter its hormone affinity state upon activation. The heterodimeric soluble guanylyl cyclase (sGC) contains a heme moiety within the N-terminal domain of the β_1 subunit. This latter domain contains the two known steps in the activation process of sGC: NO binding (1) and breakage of the Fe^{2+} -His105 bond (2). Pharmacology and Therapeutics, 104, by P. S. Padayatti, P. Pattanaik, X. Ma, and F. van den Akker. "Structural insights into the regulation and the activation mechanism of mammalian guanylyl cyclases," pp. 83–99 (2004). Adapted with permission from Elsevier.

receptors of unknown function. GC-D is expressed in rat olfactory sensory neurons [100] while GC-G has been localized to lung, intestine, and skeletal muscle [101].

Regulation of GCs by phosphorylation can either be stimulatory or mediate desensitization. Six phosphorylation sites have been identified in GC-A and five sites have been identified in GC-B localized to the KHD in both enzymes. Phosphorylation of these sites results in GC activation, and dephosphorylation at these sites desensitizes the GCs to further stimulation by agonist (reviewed in [91]). GC-C differs from GC-A and GC-B in that phosphorylation of S1029 by PKC activates this receptor [102], and GC-C desensitization is mediated by binding of SH3 domain-containing proteins [103].

3.3.4 Cellular Targets of cGMP

The cellular consequences of synthesizing the second messenger cGMP are mediated by binding of cGMP to three types of cellular substrates: PKG, CNG channels, and PDEs, reminiscent of cAMP signal transduction described above. PDEs can be regulated by either cAMP or cGMP or both or by the cognate receptors activated by the cyclic second messengers. PDE activity can be either enhanced or inhibited by cyclic nucleotide binding, creating positive- or negative-feedback loops for cAMP- and/or cGMP-mediated signaling, resulting in a point of convergence for the two signaling pathways.

PKG is a serine/threonine-directed kinase that exhibits three isoforms grouped into two types that form functional homodimers [104]. Type I PKG exhibits two splice variants I α and I β that differ only in the first 100 amino acids of the protein, are widely distributed, and are usually isolated from soluble tissue extracts [104, 105]. Type II PKG is a monomer of 86 kDa that arises from a separate gene product with an N-terminal domain that differs significantly from the type I PKGs [106]. PKG proteins contain several domains that mediate various functions of the protein. The C-terminal region contains the catalytic domain composed of the ATP and substrate binding sites and exhibits the greatest sequence similarity among the PKG isoforms. Two allosteric cGMP binding sites are located immediately N-terminal to the catalytic domain. Binding of cGMP to these domains initiates conformational changes in the protein that result in activation of enzymatic activity [107]. Upstream from the cGMP binding sites is an autophosphorylation/autoinhibitory domain that contains a pseudosubstrate sequence that binds to and inhibits the catalytic domain. Phosphorylation of sites in and around this region and/or cGMP binding activates PKG I [108, 109], where cGMP binding alone is sufficient for PKG II activation [106]. The N-terminal domain of mammalian PKGs contains six to nine heptad repeats that mediate an extended leucine zipper motif that is required for dimerization of the PKG monomers, resulting in increased affinity for cGMP binding and activation as well as interaction with other proteins [104]. PKG activation and cGMP activation are involved in a variety of cellular activities, including smooth muscle vasorelaxation, regulation of arterial blood pressure, modulation of calcium release by inositol 1,4,5-trisphosphate receptors [110] and NMDA receptor channels [111], and regulation of apoptosis and survival in neural cells [112].

CNG channels represent another cellular target for cGMP binding and regulation. The best characterized examples of CNG channel regulation by cGMP are in the visual and olfactory sensory systems. The photoreceptor cGMP-gated channel consists of 690 amino acids with six transmembrane domains located in the N-terminus and the cGMP binding domain located in the hydrophilic C-terminus, reviewed in [113]. The channel displays a low affinity for cGMP that maintains the channel in an open state due to the unusually high concentration of cGMP in photoreceptor cells (10 μ M). Hydrolysis of cGMP by the transducin-cGMP PDE system closes the channel allowing for the removal of intracellular Ca^{2+} by the $\text{Na}^+ - \text{Ca}^{2+}$ exchanger, gating the system on rapid changes in cGMP concentration. The CNG channel in olfactory epithelium can be gated by either cGMP or cAMP and requires higher concentrations of cGMP for activation than PKG requires [114].

Cyclic nucleotide second messengers are synthesized in response to a variety of cellular stimulants, including hormones, neurotransmitters, and peptides, that bind to cognate receptors that ultimately lead to activation of adenylyl and guanylyl cyclase.

The plethora of mechanisms utilized to synthesize and propagate specific cAMP and cGMP signaling is complex and diverse as discussed above, leading one to wonder how specificity is achieved with such remarkably diverse molecules. Specificity in signaling can be achieved by organizing the key players into supramolecular complexes that are contained in both space and time to direct simultaneous and interconnected signaling pathways into a distinct cellular response. Such a three-dimensional organization of signaling pathways has been the focus of considerable research interest (reviewed in [115]), and is achieved on many levels. First, signal transduction components display a particular pattern of expression within various tissues. Second, coexpression of anchoring proteins such as AKAPs can further discriminate the location of signaling molecules to specific subcellular sites and act as adapter molecules to provide a framework to both orient kinases and phosphatases towards selected substrates and corral signaling gradients. No doubt further experimentation will reveal more participants in the orchestration of cellular signaling.

3.4 PHOSPHOLIPASE C-, PROTEIN KINASE C-, AND Ca^{2+} -REGULATED SIGNALING PATHWAYS

The cellular responses elicited by the interaction of many extracellular signaling molecules with their cell surface receptors are triggered by the rapid hydrolysis of a minor membrane phospholipid, PIP_2 (Fig. 3.5). This reaction is catalyzed by phosphoinositide-specific PLC isozymes and results in the generation of two intracellular messengers, DAG and inositol 1,4,5-trisphosphate (IP_3). These messengers then promote the activation of PKC and the release of Ca^{2+} from intracellular stores, respectively. The PLC isozymes responsible for the generation of IP_3 and DAG have been classified on the basis of amino acid sequence into five major types (Fig. 3.6). These groups also differ in the mechanism by which the isozymes are activated in response to ligand interaction with various receptors. For example, $\text{PLC}\beta$ has been shown to be activated by GPCRs through $\text{G}\alpha_q$ or $\text{G}\beta\gamma$ and $\text{PLC}\gamma$ isozymes are regulated by receptor and nonreceptor tyrosine kinases.

3.4.1 IP_3 and Phosphoinositides as Signaling Molecules

IP_3 , generated from PIP_2 following agonist-dependent activation of PLC, is highly mobile and diffuses into the cytoplasm. There it releases Ca^{2+} from the endoplasmic reticulum (ER) by binding to an IP_3 receptor (IP_3R), which is an IP_3 -gated Ca^{2+} release channel in the ER membrane. IP_3 is further converted by the action of several distinct kinases and phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signaling. Finally, it is sequentially dephosphorylated to free inositol, which can be reutilized by phosphatidylinositol (PI) synthase to form PI. PI is sequentially phosphorylated to form phosphatidylinositol phosphate (PIP) and PIP_2 (Fig. 3.7).

PIP_2 not only serves as the source of DAG and IP_3 but is also a substrate of another receptor-activated enzyme, PI 3-kinase (PI3K). Phosphorylation of PIP_2 by PI3K yields phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which functions as a distinct second messenger. Signaling proteins (mostly kinases) with pleckstrin homology (PH) domains accumulate at sites of PI3K activation by directly binding

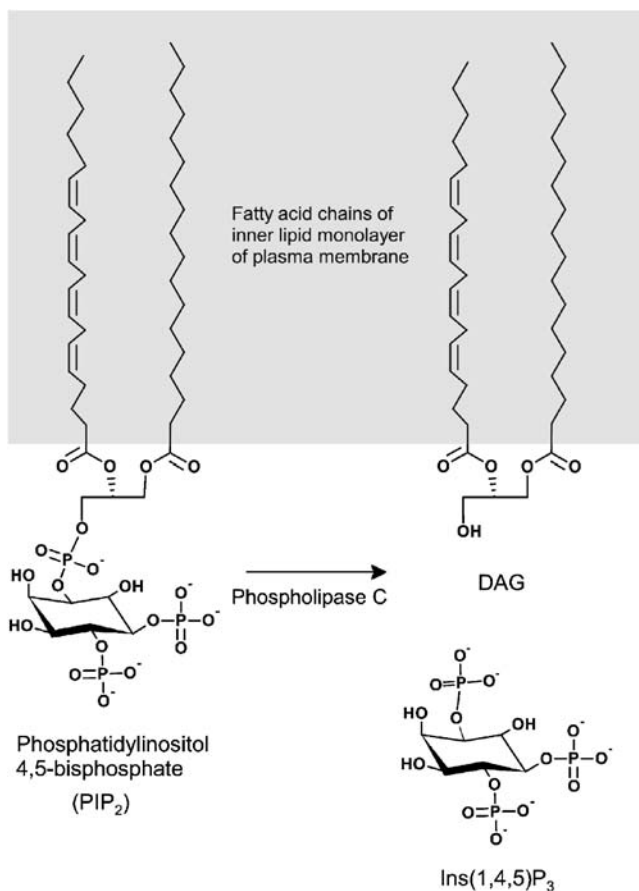


Figure 3.5 Hydrolysis of PIP₂ by phospholipase C. The structure shows phosphatidylinositol 4,5-bisphosphate PIP₂, which contains a phosphorylated inositol head group connected to a DAG via a phosphodiester linkage. The fatty acid moiety is typically stearyl-arachidonyl. Phospholipase C hydrolyzes PIP₂ into inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and DAG.

to PIP₃ [116]. The formation of PIP₃ thus provides an anchor for assembling signaling proteins at specific locations in the membrane in response to cell stimulation. These signaling proteins coordinate complex events leading to changes in cell metabolism, growth, movement, and survival.

In addition, PIP₂ is a signaling molecule in its own right [117]. High-affinity binding of PIP₂ to a number of proteins causes them to relocate to the plasma membrane, and this is typically mediated by the PH domain of phospholipases (e.g., PLC δ -1) and exchange factors of GTPase (e.g., Sos, Vov). Finally, PIP₂ regulates the organization of the actin cytoskeleton, the activities and localization of proteins involved in vesicle trafficking, the activity of many ion channels and transporters, and the recruitment of cytosolic proteins to specific membranes [118].

Lithium has been used for the treatment of bipolar disorder since the 1960s. However, the mechanism of lithium action is still unclear. Lithium inhibits inositol monophosphatase, which is an enzyme of the last common step of dephosphorylation of inositol phosphates (Fig. 3.7). All inositol monophosphate are metabolized by

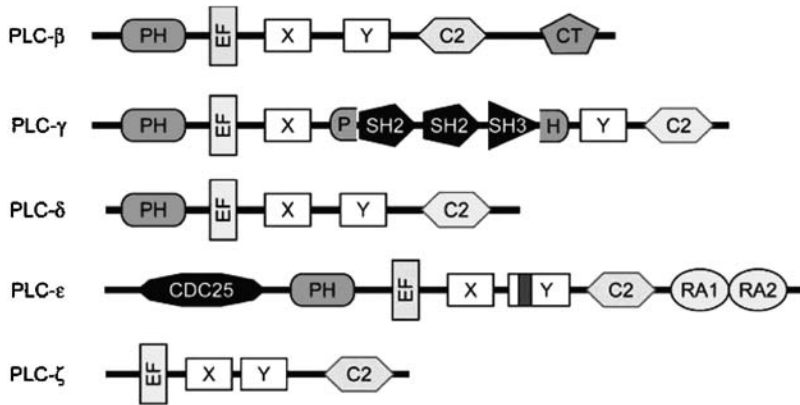


Figure 3.6 Domain structure of mammalian PLC family members. Hallmarks of PLC family members are an N-terminal PH domain, which binds PIP_2 and $\text{G}\beta\gamma$ subunits, and EF, X, Y, and C2 motifs forming the catalytic core for phosphoinositide hydrolysis. $\text{PLC}\beta$ can be activated by $\text{G}\alpha_q$ through unique C-terminal (CT) domain, which also acts as a $\text{G}\alpha_q$ GAP. Unique to $\text{PLC}\gamma$ are two Src homology 2 (SH2) domains and a Src homology 3 (SH3) domain that bisect the pleckstrin homology (PH) domain. The SH2 domains confer sensitivity to stimulation by growth factors like platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) receptors. $\text{PLC}\epsilon$ differs from the other types of isoforms in that it possesses an NH_2 -terminal Ras guanine nucleotide exchange factor (RasGEF, cdc25)-like domain and at least one and perhaps two COOH terminal Ras binding (RA) domains. $\text{PLC}\delta$ isoform may be regulated by Ca^{2+} ; however, the mechanisms by which $\text{PLC}\delta$ enzymes couple to and are regulated by the membrane receptor is less clear. $\text{PLC}\zeta$, the most recently identified PLC isoform, is reportedly responsible for sperm-mediated Ca^{2+} oscillations that occur during fertilization. (From [2] with permission from Birkhäuser Publishing, Basel, Switzerland.)

inositol monophosphatase. The de novo synthesis of *myo*-inositol also occurs by isomerization of glucose 6-phosphate to form IP_1 . Thus in the presence of lithium, these monophosphates cannot be dephosphorylated to yield free inositol, which is required to regenerate PIP_2 . Because *myo*-inositol is essential for the formation of PIs, it is hypothesized that lithium's therapeutic action is mediated by reducing levels of inositol in the brains of patients with bipolar disorder [119]. While the inositol depletion hypothesis still offers an attractive mechanism of lithium efficacy, its validity remains to be demonstrated [120].

3.4.2 Ca^{2+} as a Signaling Molecule

Calcium (Ca^{2+}) is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. Cells at rest have a Ca^{2+} concentration of 100 nM but are activated when this level rises to roughly 1 μM . Cells generate their Ca^{2+} signals by using both internal and external sources of Ca^{2+} . There are several pathways for the entry of external Ca^{2+} into the cell: (1) Voltage-operated Ca^{2+} channels, which are plasma membrane ion channels, are activated by membrane depolarization. (2) Receptor-operated Ca^{2+} channels open in response to receptor activation by extracellular ligand, such as nicotinic acetylcholine receptor and NMDA receptor. (3) Store-operated Ca^{2+} channels open in response to depletion

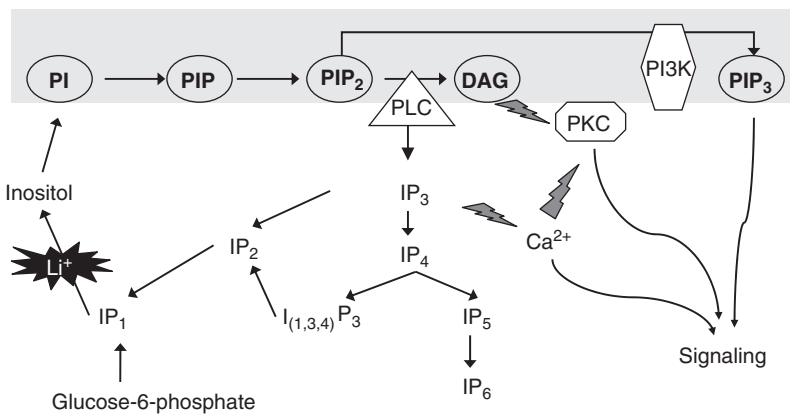


Figure 3.7 Biosynthesis and metabolism of phosphoinositide signals. Inositol is transferred to DAG and phosphorylated to form PIP₂ in the membrane. Cell stimulation activates PLC to release DAG and the soluble second messenger Ins(1,4,5)P₃. Ins(1,4,5)P₃ diffuses through the cytosol and releases Ca²⁺ from the ER and DAG remains in the membrane and activates PKC. Activation of PI3K by extracellular stimuli also phosphorylates PIP₂ into PIP₃, which regulates many signaling enzymes. Ins(1,4,5)P₃ is further phosphorylated to form the higher order InsP (InsP₄, InsP₅, and InsP₆) or dephosphorylated and recycled into inositol. All inositol monophosphates (InsP₁) are metabolized by inositol monophosphatase, an enzyme that is inhibited by lithium (Li⁺). The de novo synthesis of *myo*-inositol also occurs by isomerization of glucose 6-phosphate to form InsP₁. Thus in the presence of lithium, these monophosphates cannot be dephosphorylated to yield free inositol, which is required to regenerate PIP₂. Lithium also inhibits inositol polyphosphate-1-phosphatase, which hydrolyzes Ins(1,3,4)P₃ to Ins(3,4)P₂ and Ins(1,4)P₂ to Ins4P.

of internal Ca^{2+} stores. The mechanism by which those channels “sense” the filling status of the intracellular pool is unknown. The internal stores are held within the membrane systems of the ER or the equivalent organelle, the sarcoplasmic reticulum (SR) of muscle cells. Release from these internal stores is controlled by various channels, of which the IP_3R and ryanodine receptor (R YR) families have been studied most extensively. Intracellular messenger IP_3 , produced by the hydrolysis of PIP_2 by a family of PLC, binds IP_3R and releases Ca^{2+} from the ER. Most of the Ca^{2+} that enters the cytoplasm is rapidly bound to various cytosolic buffers such as parvalbumin, calbindin, and calretinin. Cytosolic buffers are involved in the shaping and duration of Ca^{2+} signals and also limit the spatial spreading of local Ca^{2+} signals [121]. This is particularly important in neurons that contain high concentrations of buffers, such as parvalbumin and calbindin, which ensure that Ca^{2+} signals are largely confined to synapses. Once Ca^{2+} has carried out its signaling functions, it is rapidly removed from the cytoplasm by various pumps and exchangers. The plasma membrane Ca^{2+} -ATPase pumps and Na^{+} - Ca^{2+} exchangers extrude Ca^{2+} to the outside whereas the sarco-ER ATPase pumps return Ca^{2+} to the internal stores.

3.4.3 DAG Activates Protein Kinase C

At the same time that the IP₃ produced by hydrolysis of PIP₂ is increasing the concentration of Ca²⁺ in the cytosol, the other cleavage product of PIP₂—DAG—is

exerting different effects. The hydrophobic DAG that remains within the plane of the plasma membrane functions as a second messenger by activating PKC. In the absence of hormone stimulation, PKC is present as a soluble cytosolic protein that is catalytically inactive [122]. A rise in the cytosolic Ca^{2+} level causes PKC to bind to the plasma membrane, where it can be activated by the membrane-associated DAG. In addition to DAG and Ca^{2+} , the enzyme requires phosphatidylserine (PS). Thus activation of PKC depends on both Ca^{2+} ions and DAG, suggesting an interaction between the two branches of the inositol-lipid signaling pathway. Prolonged activation of PKC can be accomplished with phorbol esters, tumor-promoting compounds that activate PKC by mimicking DAG. PKC is also regulated by autophosphorylation and transphosphorylation on tyrosine and/or serine/threonine residues by upstream kinases. Phosphorylation of PKC by these kinases induces maturation of the PKC enzyme as well as activating its enzymatic activity.

The PKC families are divided into three groups according to their regulatory properties [123, 124] (Fig. 3.8). Conventional PKC isozymes (α , γ , and the alternatively spliced βI and βII) are stimulated by DAG, PS, and Ca^{2+} ; novel PKC isozymes (δ , ϵ , η , θ) are stimulated by DAG and PS; atypical PKC isozymes (ς , ι/λ) are stimulated by PS and do not respond directly to either DAG or Ca^{2+} . Since the multiple PKC isoforms are discrete functional entities with distinct cellular and subcellular localizations, it is likely that they possess unique patterns of sensitivity in input signals. In the nervous system, activation of PKC has been related to enhancement of neurotransmitter release, regulation of ion channels, control of growth and differentiation, and modification of neuronal plasticity. The mechanism of action of PKC and the physiological substrates of PKC in controlling these neural functions are still poorly characterized. Identification of specific substrates for each PKC species at different cellular and subcellular locations is essential to understanding the physiological functions of these enzymes.

3.4.4 Action of Ca^{2+} and Its Mediator Calmodulin

Ca^{2+} is an almost universal intracellular messenger, controlling a diverse range of cellular processes, such as gene transcription, muscle contraction, neurotransmitter or hormone release, and cell proliferation. How do Ca^{2+} signals produce changes in cell function? The information encoded in transient Ca^{2+} signals is deciphered by various intracellular Ca^{2+} binding proteins that convert the signals into a wide variety of biochemical changes. Ca^{2+} binding proteins can be divided into Ca^{2+} buffers (described above) and Ca^{2+} sensors on the basis of their main functions. The latter respond to an increase in Ca^{2+} by activating diverse processes. Some Ca^{2+} sensors, such as PKC, bind to Ca^{2+} and are directly regulated in a Ca^{2+} -dependent manner. Other Ca^{2+} -sensor proteins, however, are intermediaries that couple the Ca^{2+} signals to biochemical and cellular changes. The most prominent examples of sensors include troponin C (a protein dedicated to regulating cardiac and skeletal muscle contraction) and the multifunctional Ca^{2+} transducer calmodulin (CaM).

CaM is used more generally to regulate many processes such as the contraction of smooth muscle cells, crosstalk between signaling pathways, gene transcription, ion channel modification, and metabolism. CaM is a ubiquitous, multifunctional Ca^{2+} binding protein with a molecular weight of approximately 17 kDa. The binding of Ca^{2+} enhances CaM's affinity for most of its target proteins. These changes in

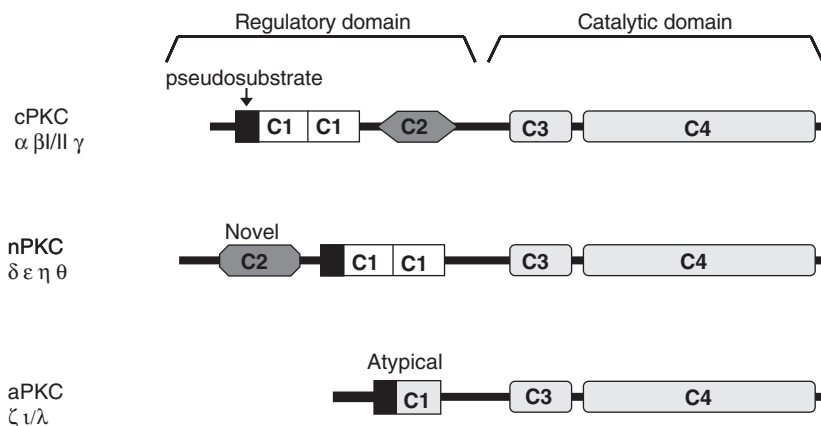


Figure 3.8 Domain structure of mammalian PKC family members. All PKCs contain a C-terminal catalytic domain and an N-terminal regulatory domain. The catalytic domain is highly conserved in PKC family members and is comprised of two regions: the ATP binding lobe (C3) and the substrate binding lobe (C4). It is maintained in an inactive state by an autoinhibitory sequence in the regulatory moiety, the pseudosubstrate (black box), that sterically blocks the active site. The regulatory domains are more diverse and define three classes of PKC. Conventional PKC (cPKC) isoforms have a Ca^{2+} binding C2 domain and tandem DAG binding C1 domains, conferring a dependence on both Ca^{2+} and DAG for high-affinity membrane binding and maximal kinase activity. Novel (nPKC) isoforms have a novel variant of the C2 domain that does not bind Ca^{2+} and two conventional C1 domains; these isoforms are DAG dependent but Ca^{2+} independent. Atypical (aPKC) isoforms have no C2 domain and a single C1 domain that does not bind DAG, and so are Ca^{2+} and DAG independent; atypical PKC isoforms depend only on phosphatidylserine and phosphorylation for activation. The role of the novel C2 domain in nPKCs and that of the atypical C1 domain in aPKCs is not clear, but each may regulate the subcellular distribution of these isoforms through protein–protein interactions. (Adapted from [125a].)

affinity occur because Ca^{2+} binding induces a conformational change in CaM. Studies on crystal structure show that CaM is a symmetrical dumbbell-shaped molecule with two Ca^{2+} binding domains on each end connected by an α -helical structure [125]. The K_d for the binding of the four Ca^{2+} molecules to CaM are in the micromolar range, with two higher and two lower affinity sites. Ca^{2+} -dependent binding of CaM to many proteins or enzymes would occur primarily in response to cell activation as opposed to resting Ca^{2+} levels, although CaM can bind several proteins in the absence of Ca^{2+} . Thus, hormones and neurotransmitters that elevate intracellular Ca^{2+} can activate CaM-dependent processes that will contribute to the physiological responses. Moreover, Ca^{2+} binding to the low-affinity sites of CaM is greatly enhanced in the presence of a CaM binding enzyme [126]. The affinity for Ca^{2+} of each CaM binding enzyme complex is unique to that complex. Therefore, different enzymes can have different Ca^{2+} sensitivities, despite being activated through the same CaM.

The Ca^{2+} –CaM complex stimulates a wide array of enzymes, pumps, and other target proteins. Interestingly, many of the most highly characterized effectors are directly (protein kinase/phosphatase) or indirectly (adenylyl cyclase/phosphodiesterase) involved in protein phosphorylation [127]. For example, calmodulin kinase II

(CaMKII) is a major target of the Ca^{2+} /CaM second-messenger system. These enzymes regulate fuel metabolism, ionic permeability, neurotransmitter synthesis, neurotransmitter release, and learning and memory. Once bound to Ca^{2+} /CaM, the multimeric CaMKII is released from its autoinhibitory status and maximally activated, which then leads to an intraholoenzyme autophosphorylation reaction. Autophosphorylation of CaMKII makes it independent of Ca^{2+} and renders it constitutively active and, in essence, it is the memory of a previous calcium pulse. Calcineurin (also known as protein phosphatase 2B) is another major target protein that is activated by Ca^{2+} /CaM. Calcineurin is a serine–threonine phosphatase that consists of a heterodimeric protein complex composed of a catalytic subunit (CNA α or CNA β) and a regulatory subunit (CNB1). Upon activation, calcineurin directly binds to and dephosphorylates the nuclear factor of activated T cells (NF-AT) transcription factors within the cytoplasm allowing them to translocate to the nucleus and participate in the regulation of gene expression. Ca^{2+} /CaM can also regulate intracellular cAMP levels through two opposite reactions: activation of adenylyl cyclase 1 and 8 or stimulation of some of the cAMP phosphodiesterases; these enzymes degrade cAMP and terminate its effects. Those reactions thus link Ca^{2+} and cAMP, one of many examples in which these two second messengers interact to fine tune certain aspects of cell regulation. The plasma membrane Ca^{2+} –ATPase pump, various ion channels, the ryanodine receptor, and isoforms of the IP₃ receptor are other important targets of Ca^{2+} /CaM.

3.5 PROTEIN PHOSPHORYLATION

Protein phosphorylation is a prominent feature of many signaling pathways. The reversible covalent attachment of a phosphate group to cellular proteins can have profound effects on the protein's activities and properties. The ability of a cell to phosphorylate and dephosphorylate a substrate is intricately regulated by the interplay of protein kinases and protein phosphatases. Protein kinases and phosphatases are broadly divided into groups based on their abilities to phosphorylate specific amino acids (i.e., serine/threonine or tyrosine), although this classification is not exclusive and in fact several kinases and phosphatases exhibit dual specificity. This section will focus on several families of kinases and phosphatases that are critical for the control of many processes that include cellular proliferation, differentiation, apoptosis, and synaptic plasticity.

3.5.1 Protein Tyrosine Kinases

Protein tyrosine kinases (PTKs) catalyze the transfer of phosphate to tyrosine residues in polypeptide. PTKs are divided into two main classes: receptor and non receptor tyrosine kinases. Receptor tyrosine kinases (RTKs) are monomeric transmembrane proteins with an intracellular catalytic kinase domain and an extracellular ligand binding domain. Examples of RTKs include the epidermal growth factor receptor (EGFR) family, neurotrophin receptor family, and Eph receptor family.

RTKs generally reside within a cell in an unphosphorylated monomeric conformation. Activation of RTKs requires ligand binding to the extracellular domain and dimerization of two ligand-bound receptors (Fig. 3.9). However, RTK

activation is not limited to homodimerization and crosstalk between heterodimers of related RTK families has been reported [128]. The interaction of adjacent intracellular RTK domains disrupts the autoinhibitory constraints and permits the transphosphorylation of tyrosine residues within their cytoplasmic regions. Once activated, the phosphotyrosine residues of RTKs provide docking sites for the recruitment of signaling proteins to the cell membrane, leading to the propagation of multiple signaling cascades [129]. Many signaling proteins recruited to RTKs contain SH2 (src-homology 2) and/or PTB (phosphotyrosine binding) domains [130]. These domains recognize and bind to phosphotyrosine residues and their surrounding structures. A diverse array of SH2- containing proteins, ranging from scaffolding

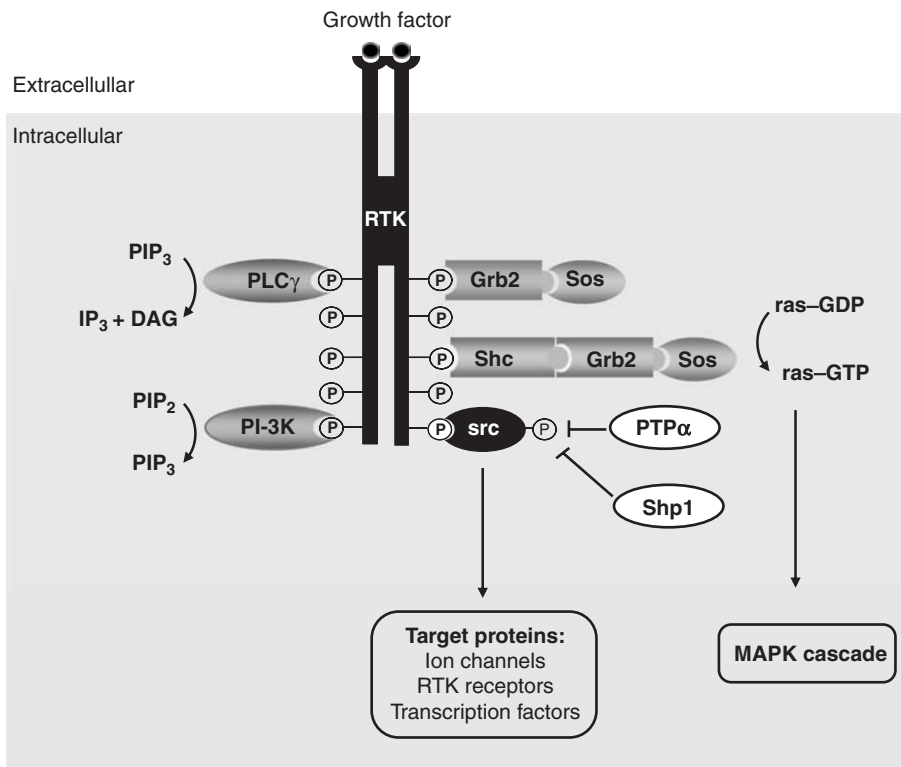


Figure 3.9 Receptor and nonreceptor tyrosine kinase signaling pathways. Dimerization of ligand-bound receptor RTKs activates their kinase domain resulting in transphosphorylation. Phosphotyrosine residues of RTKs recruit SH2-containing proteins such as the adapter proteins Grb2 and Shc. Association of Sos with Grb2 positions Sos in proximity to Ras. Sos activates Ras by exchanging GDP for GTP. Ras-GTP activates the MAPK cascade. The SH2-containing enzymes PLC γ and p85 (the regulatory domain of PI-3 kinase) also associate with phosphotyrosine residues of RTKs and are activated either by tyrosine phosphorylation or release of autoinhibitory constraints, respectively. PLC γ catalyzes the hydrolysis of PIP_2 and the generation of IP_3 and DAG. PI-3 kinase catalyzes the phosphorylation of PIP and PIP_2 to generate PIP_2 and PIP_3 , respectively. The SH2 domains of the nonreceptor tyrosine kinase Src also associate with activated RTKs. Src is activated by both dephosphorylation and phosphorylation events mediated in part by the phosphatases $PTP\alpha$ /Shp1 and kinase activity of the EGF receptor.

molecules to enzymes, can associate with activated RTKs and elicit multiple signaling pathways. For example, PLC γ and the regulatory domain of PI-3 kinase (p85) bind to phosphotyrosine residues of PDGF receptors and are activated by tyrosine phosphorylation or release of an autoinhibitory constraint, respectively. Similarly, the adapter proteins Grb2 and Shc also associate with the phosphotyrosine residues of RTKs and activate several signaling pathways such as the Ras-MAP kinase cascade [129].

The nonreceptor tyrosine kinases (nRTKs) lack transmembrane domains and are localized in the cytosol, inner surface of the plasma membrane, and nucleus. They include members of the Abl, Src, JAK, FAK, and Syk families. The Src family is the largest family of nonreceptor tyrosine kinases and is comprised of nine members, including Src itself [131].

Regulation of the Src family kinases is intricate and dependent on the interplay between kinases, phosphatases, and protein–protein interactions via their multiple SH domains [132]. SH1 is the catalytic domain of Src and is maintained inactive by impeding substrate access. Substrate accessibility is blocked by several intramolecular mechanisms that include positioning of a key regulatory tyrosine within the catalytic domain and binding of a constitutively phosphorylated tyrosine residue in the carboxy terminus to an upstream SH2 domain. Substrate access to the catalytic site of Src requires both phosphorylation and dephosphorylation events, specifically dephosphorylation of the phosphotyrosine residue associated with SH2 binding and phosphorylation of the regulatory tyrosine within the kinase domain [133, 134].

The subcellular localization of Src regulates its catalytic activity. The trafficking of Src to the plasma membrane and association with specific membrane proteins is critical for controlled kinase activity. Src tethers to the plasma membrane by covalently attached lipid chains that position the kinase in proximity to transmembrane receptors such as the EGFR [135]. The SH2 domain of Src associates with the phosphotyrosine residues of the EGFR (Fig. 3.9). The EGFR activates Src by phosphorylating key tyrosine residues. Once activated, Src phosphorylates many target proteins that include the EGFR, ion channels, synaptic vesicle proteins, and transcription factors [136–139].

3.5.2 Mitogen-Activated Protein Kinases

The MAPKs are a family of serine/threonine kinases that catalyze the transfer of phosphate to serine and threonine residues within a specific protein. MAPKs propagate a cascade of intracellular phosphorylation events in response to a variety of extracellular signaling stimuli. To date, four MAPK cascades have been identified in mammalian cells. They are the ERK1/ERK2 (extracellular signal-regulated kinase), ERK5 (or big MAPK), JNK/stress-activated protein kinase (SAPK), and p38 kinase cascades [140] (Fig. 3.10). Each MAPK cascade displays a high degree of specificity and in general is functionally separate from the others.

The ERK1/ERK2 cascade is activated in response to cues that promote proliferation and differentiation, such as growth factors and the activation of their receptors [141]. Crosstalk between growth factor receptors, such as the EGFR, and the ERK1/2 kinase cascade is relayed by a number of adapter/scaffolding molecules that include the previously mentioned Grb2. Association of Grb2 with the phosphorylated EGFR promotes the recruitment of Sos (a guanine nucleotide binding protein) to the plasma

membrane, positioning it in close proximity to membrane-bound Ras [142, 143]. Sos activates Ras-GDP by exchanging GDP for GTP. Ras-GTP activates Raf, a MAP kinase kinase kinase (MKKK). Raf is a serine/threonine kinase that phosphorylates MEKK, a MAP kinase kinase (MKK). Raf phosphorylates MEK on two serine residues within the consensus sequence S-X-X-X-S/T resulting in MEK activation. MEK is a dual-specificity enzyme that activates the MAP kinase, ERK1/2, by phosphorylating both threonine and tyrosine residues in the consensus sequence TEY (reviewed in [140]). Once activated, ERK1/2 translocates to the nucleus and phosphorylates several target proteins, including the transcription factor Elk-1 (Fig. 3.10) [144]. ERK1/2 activity is not restricted to nuclear targets and includes cytoskeletal proteins, cytoplasmic kinases, and growth factor receptors [145–148].

The JNK/SAPK and p38 kinase cascades respond mainly to stress signals, such as ultraviolet irradiation and proinflammatory cytokines, whereas the ERK5 pathway can transmit both mitogenic and stress signals. The ERK5, JNK/SAPK, and p38 kinase cascades follow the same principles of the ERK1/ERK2 pathway involving a series of consecutive phosphorylation steps that ultimately result in activation of a specific MAPK (Fig. 3.10). Once phosphorylated and activated, ERK5, JNK/SAPK, and p38 can translocate to the nucleus and regulate the activity of transcription factors (Fig. 3.10). Similar to ERK1/2, JNK/SAPK and p38 can phosphorylate several cytosolic proteins [140].

Although each MAPK cascade shows a high degree of specificity and functional separation, crosstalk between these pathways has been reported. For example, MEK1/MEK2 and MKK3 are the only known activators of ERK1/2 and p38, respectively [149, 150]; however, MKK4 is capable of activating both the JNK/SAPK and p38 pathways [150, 151].

3.5.3 G-Protein-Coupled Receptor Kinases

G-protein-coupled receptor kinases (GRKs) phosphorylate serine/threonine residues of agonist-occupied GPCRs and play a key role in GPCR desensitization (homologous desensitization). To date, seven mammalian genes encoding GRKs (GRK 1–7) have been identified. The majority of GRKs are ubiquitously expressed with the exception of GRK4 and GRK1/7. GRK4 is expressed in the testis, brain, myometrium, and kidney. In contrast, GRK1/7 are exclusively expressed in the retina [152].

Cellular compartmentalization is an important feature of regulated GRK signaling. This is achieved through several distinct mechanisms that are GRK-subtype specific. For example, posttranslational lipid attachment and modification to the carboxyl terminus of GRK1 and GRK4/6 is required for membrane localization and positioning of the GRK in close proximity to the receptor substrate [153–155]. Alternatively, some GRKs (i.e., GRK2 and GRK3) are ferried to the plasma membrane by a cooperative attachment to $\beta\gamma$ subunits that are released from the G-protein trimer upon activation of a GPCR [156, 157].

The molecular events underlying GRK-mediated GPCR phosphorylation, desensitization, and internalization occur in a sequential manner (Fig. 3.11). GRKs associate exclusively with the active (agonist-bound) GPCR conformation. GRK association with its activated receptor is an important initial step for GRK-mediated receptor phosphorylation and can be impaired or enhanced by the association of Ca^{2+} binding proteins, autophosphorylation or PKC phosphorylation of specific GRK subtypes.

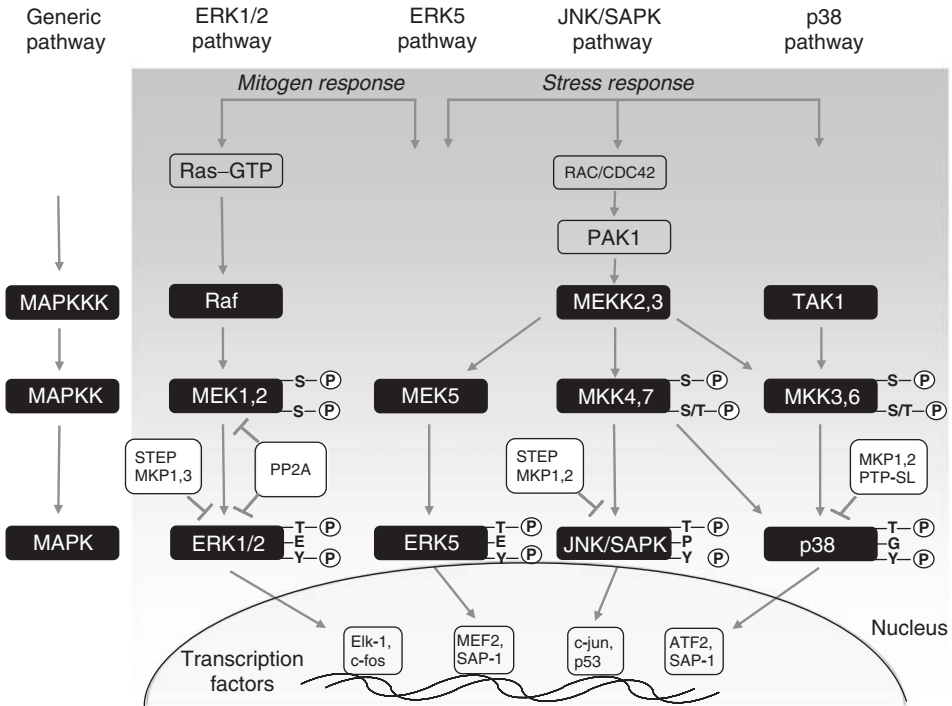


Figure 3.10 Schematic representation of MAPK cascades. A variety of signals trigger mitogenic or stress responses and these signals are transmitted to the interior of the cells where they activate appropriate cascades. Each of the four MAPK cascades (ERK1/2, ERK2, JNK/SAPK, and p38) consists of a three-enzyme module that includes MAPKKK, MAPKK, and MAPK (black boxes). These cascades are inhibited by phosphatases (white boxes).

The association of the Ca^{2+} binding proteins calmodulin and recoverin generally impairs receptor–GRK association. For example, calmodulin specifically inhibits GRK2/5–receptor association whereas recoverin specifically inhibits GRK1 [158, 159]. GRK1 and GRK5 are autophosphorylated, a modification that also impairs receptor interactions [160, 161]. In particular, GRK1 autophosphorylation inhibits its association with the phosphorylated receptor substrate—a mechanism believed to facilitate dissociation of the GRK–receptor complex following receptor phosphorylation [162]. GRK2 and GRK5 are also substrates for PKC phosphorylation, although the functional consequences are different for the two subtypes. GRK2 activity is enhanced by PKC phosphorylation whereas GRK5 activity is reduced [163, 164].

Generally, GRKs phosphorylate serine/threonine residues within the intracellular loops and/or the C-terminus of GPCRs. Although a GRK-specific phosphorylation consensus sequence has not been identified, GRK1 and GRK2 preferentially phosphorylate serine/threonine residues flanked by acidic residues on the carboxyl or amino terminal sides, respectively [165]. GRK5 and GRK6, on the other hand, phosphorylate serine residues that are downstream from basic amino acids [166, 167]. The phosphorylated receptor–GRK complex provides a platform for the association of arrestin. Arrestin association to the receptor–GRK complex prevents the exchange

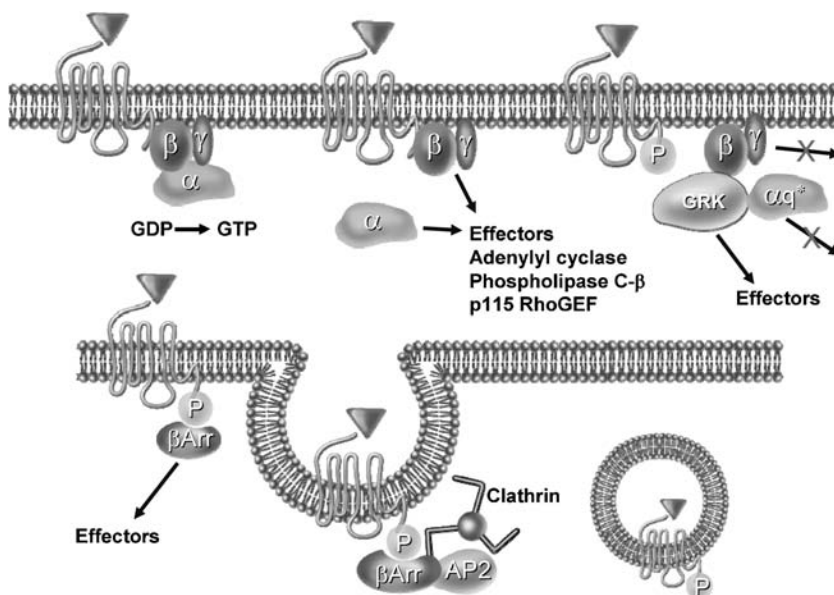


Figure 3.11 Schematic diagram representing GRK-mediated regulation of GPCRs. Following agonist binding, GPCRs activate heterotrimeric G proteins that in turn couple to effectors such as adenylyl cyclase, PLC β , and p115 RhoGEF. GRKs phosphorylate the agonist-bound form of the receptor and uncouple GPCRs from G proteins. The phosphorylated receptor recruits arrestin, further hindering association with G proteins. AP2 and clathrin associate with the receptor/arrestin complex and internalize the phosphorylated receptor into clathrin-coated pits. (Reproduced from [168].)

of GTP for GDP on the G-protein α subunit and desensitizes the receptors response. Subsequent association with the adapter protein AP2 and clathrin facilitates the internalization of desensitized receptors into clathrin-coated pits (reviewed in [168]).

In addition to receptor desensitization and internalization, GRKs and arrestins act as adapter molecules in their own right, shuttling additional signaling proteins to activated receptors, ultimately tethering GPCR signaling to noncanonical pathways [168].

3.5.4 Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) hydrolyze phosphotyrosine residues of a polypeptide. The tyrosine-specific phosphatases are subdivided into two groups: the receptor like PTPs (RPTPs) and the intracellular PTPs.

To date, eight RPTP subgroups have been identified based on sequence motifs in their diverse extracellular domains [169]. RPTPs usually possess two intracellular phosphatase domains, a transmembrane domain and a highly variable extracellular domain. The first phosphatase domain (membrane proximal) is responsible for >90% of catalytic activity, while the second phosphatase domain binds multiple downstream partners [170]. Although RPTPs are capable of forming homodimers and heterodimers with other RPTPs, they are catalytically active as monomers and dimerization in some cases inhibits their activity by preventing substrate access [171].

PTP α is an RPTP expressed in the brain and kidney that regulates the activity of several proteins, including the tyrosine kinase Src and the adapter protein Grb2 (Fig. 3.12). The SH2 domain of Grb2 associates with a constitutively phosphorylated tyrosine residue of PTP α . Upon PKC δ phosphorylation of PTP α , Grb2 is exchanged for Src, whose SH2 domain associates with the phosphotyrosine residues of PTP α . Through a phosphotyrosine displacement mechanism, the inhibitory C-terminal phosphotyrosine residue of Src is released and dephosphorylated by PTP α , thus activating Src. Grb2 is subsequently released from PTP α and free to associate with Sos via its SH3 domain [172, 173].

Intracellular tyrosine phosphatases, in contrast to RPTPs, lack transmembrane and extracellular domains and possess only one phosphatase domain. The intracellular tyrosine phosphatases are subdivided into two groups: tyrosine specific that include SHP (src homology protein) 1 and 2 and STEP (striatal enriched phosphatase).

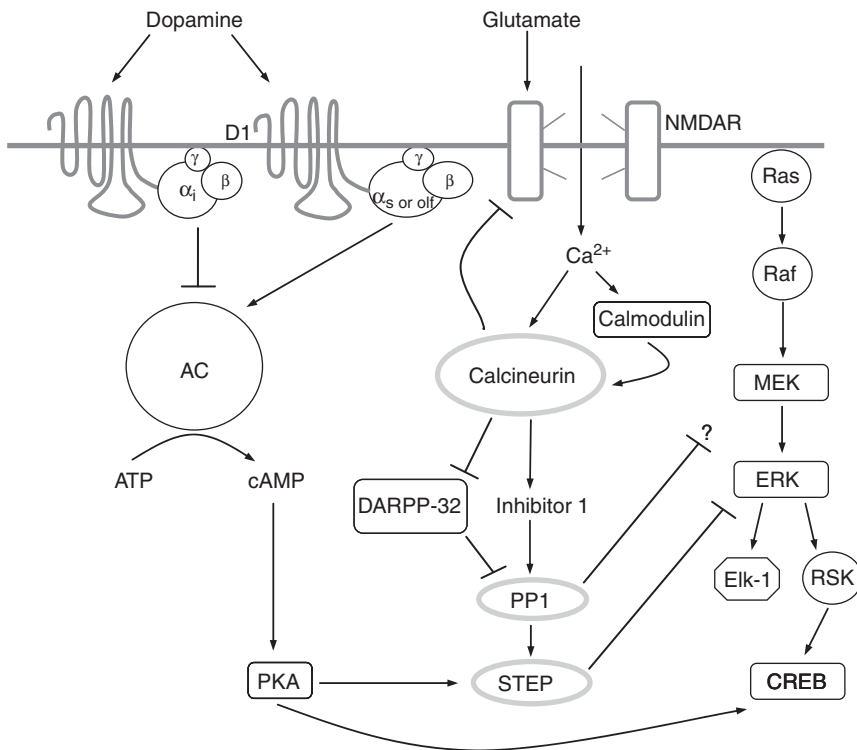


Figure 3.12 Role of phosphatases in dopamine and NMDA signaling pathways. Both glutamatergic and dopaminergic systems interact with DARPP-32. The D₁ dopamine receptor activates PKA by increasing cAMP levels. PKA phosphorylates dopamine- and CAMP-regulated phosphoprotein (DARPP-32) converting it to a potent inhibitor of the phosphatase PP1. Conversely, activation of the D₂ dopamine receptor reduces PKA-dependent phosphorylation of DARPP-32 by decreasing cAMP levels. DARPP-32 activity is also regulated by calcineurin. A major source of calcium fluctuation in the cell is through the opening and closing of NMDA receptors. Calcium activates calcineurin both directly and through interactions with calmodulin. Calcineurin indirectly activates PP1 activity by dephosphorylating and inactivating DARPP-32 and inhibitor 1. A target of PP1 activity is the tyrosine phosphatase STEP. (Reproduced from [189].)

tase) and dual-specificity phosphatases such as dsMKPs (mitogen-activated protein kinase phosphatases) and PTEN (phosphatase and tensin homolog). In general, the dual-specificity phosphatases hydrolyze phosphorylated serine, threonine, and tyrosine residues. However, PTEN is more unusual and can dephosphorylate both phosphotyrosine and the lipid phosphatidylinositol 3,4,5-triphosphate [174].

The dsMKPs are important regulators of the MAPK cascades. They inactivate members of the MAPK pathways by dephosphorylating both tyrosine and threonine residues and display unique yet overlapping substrate specificities (Fig. 3.10). For example, MKP1 dephosphorylates ERK, JNK, and p38 whereas MKP2 is selective for JNK and ERK. Even more selective is MKP3, whose target kinase is only ERK1/2 [175]. Selectivity of MKP activity is accomplished, in part, through protein–protein interactions that are independent from the phosphatase domain. In particular, a kinase interaction motif (KIM) located at the amino terminal of MKP3 directly and specifically interacts with the ERK1/2. Furthermore, MKP3 is activated by ERK1/2 [176, 177].

The regulation of MKPs is complex. Some are constitutively active (MKP3) whereas others are induced in response to specific cues (MKP1 and MKP2). Moreover, the expression of some MKPs is restricted to specific subcellular compartments; MKP3 is exclusively cytosolic versus MKP1 expression in the nucleus. MKP activity is also regulated by phosphorylation. For example, direct phosphorylation of MKP1 by ERK reduces MKP1 ubiquitination and proteasomal degradation [175].

The tyrosine phosphatase STEP also regulates the activity of the MAPK members ERK1/2 and p38 (Fig. 3.10). Similar to MKP3, the KIM domains of STEP bind ERK1/2 and inactivate the kinase's activity by dephosphorylating the regulatory tyrosine residue [178]. The enzymatic activity of STEP in neurons is regulated by phosphorylation and dephosphorylation events that are dictated in part by the dopamine D₁ receptor and glutamate NMDA receptor, respectively [179, 180]. Specifically, PKA-dependent phosphorylation of the regulatory serine within the KIM domain of STEP sterically prevents interactions with ERKs. Conversely, PP1-dependent dephosphorylation activates STEP. The regulation of phosphatases by the dopaminergic and glutaminergic pathways is described in greater detail below.

3.5.5 Serine/Threonine Phosphatases

Protein phosphatases that catalyze the dephosphorylation of serine and threonine residues have been classified into four major subtypes based on biological characteristics, sensitivities to specific inhibitors, and substrate specificities: PP1, PP2A, PP2B (also known as calcineurin), and PP2C. Other serine/threonine phosphatases identified include PP4, PP5, PP6, and PP7. PP1, PP2A, and PP2B are composed of catalytic and regulatory subunits. In contrast, PP2C exists as a monomer that is devoid of regulatory subunits and dependent on Mg^{2+} and Mn^{2+} [181].

Multiple mechanisms tightly regulate the activity, substrate specificity, and intracellular localization of protein phosphatases. In many instances, the catalytic subunits are associated with regulatory/targeting proteins that ultimately dictate the subcellular localization and substrate specificity of a phosphatase. Several phosphatase-interacting proteins have been identified that are important for localizing phosphatases in close proximity to their target proteins. For example, AKAP

functions as a scaffolding molecule that tethers calcineurin to the NMDA receptor [182]. PP1, on the other hand, is localized to neurotransmitter receptors such as the dopamine D₂ receptor by association with the PDZ domain containing the protein spinophilin [183]. In addition to proteins that are involved in the correct localization of phosphatases, several endogenous phosphatase activators/inhibitors have been identified that play an important role in phosphatase activity. The catalytic subunits of PP1 and PP2A are promiscuous, displaying high activity and low substrate specificity, and are regulated by inhibitors specific to PP1 (DARPP-32, inhibitor 1/PP1) or PP2A (inhibitor 1/PP2A) [184, 185]. In contrast to PP1 and PP2, calcineurin activity is dependent on the association of calmodulin and Ca²⁺ with its regulatory domains. Specifically, Ca²⁺/calmodulin and Ca²⁺ bind to calcineurin, releasing the autoinhibitory domain from the active site and allowing substrate accessibility [186]. However, the association of immunophilins with immunosuppressive drugs, such as cyclosporin and FK-506, inhibits calcineurin activity [187].

The interplay of phosphatases, substrates, and inhibitors/activators is illustrated by the DARPP-32 integration of dopaminergic and glutaminergic signaling (Fig. 3.13) [188, 189]. As mentioned previously, DARPP-32 is a potent inhibitor of PP1. DARPP-32 activity is regulated by phosphorylation and dephosphorylation events that are dictated by the upstream signaling pathways of the D₁ receptor and NMDA receptor. D₁ receptor activation increases cAMP levels and in turn activates PKA. PKA phosphorylates DARPP-32 at Thr³⁴, converting it to a high-affinity inhibitor of PP1 [190]. Conversely, phosphorylation by cdk5 (cyclin-dependent kinase 5) at Thr⁷⁵ inhibits the PKA-dependent phosphorylation of Thr³⁴ and dampens the inhibitory effect of DARPP-32 on PP1 activity [191]. Calcineurin also plays a pivotal role in regulating the crosstalk between the dopamine and glutamate signaling pathways. Glutamate activation of NMDA receptor channels increases Ca²⁺ influx, providing a source of Ca²⁺

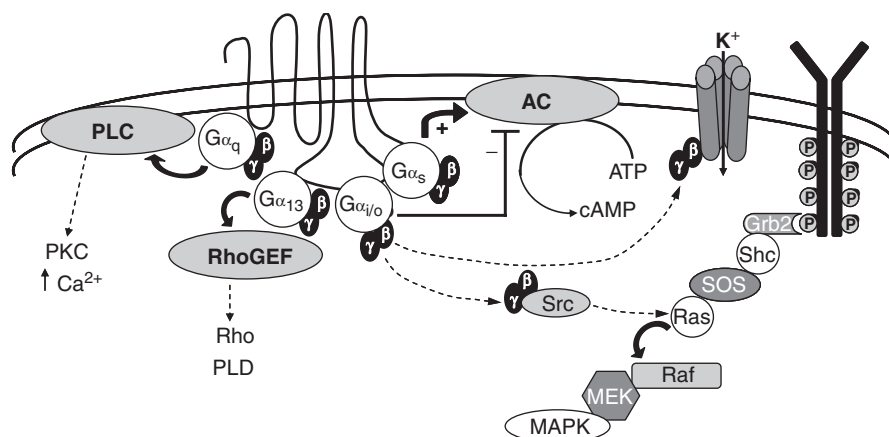


Figure 3.13 Crosstalk between signaling pathways. Following agonist stimulation, GPCRs can initiate diverse signaling pathways via coupling to different G proteins. Each of these G proteins has classical signaling molecules and corresponding downstream responses associated with it. In addition to these traditional pathways, G proteins can also regulate ion channels (Gβγ opening K⁺ channels) and proteins involved in RTK signaling (activation of Src to stimulate Ras and MAPK pathway).

for the activation of calcineurin. Once activated, calcineurin dephosphorylates DARPP-32 at Thr³⁴ and represses the DARPP-32-dependent inhibition of PP1 [192].

Regulated phosphorylation and dephosphorylation are fundamental events that control many cellular processes. It is therefore not surprising that aberrant/dysfunctional phosphorylation has significant physiological effects associated with a myriad of disease states that include cancer, diabetes, hypertension, and rheumatoid arthritis to mention a few. Many of the kinases and phosphatases discussed above are proposed to be suitable therapeutic targets. In fact, drugs such as Gleevec and Erlotinib that inhibit the tyrosine kinases ABL and the EGFR, respectively, have proven to be relatively successful in treating certain types of cancers [193, 194].

3.6 CONCLUSION

By interacting with GPCRs, RTKs, or ligand-gated ion channels, ligands are able to elicit a wide variety of responses through their signaling molecules, often in a cell-dependent manner. In recent years, the roles for many of these signaling molecules have been expanded to include novel functions. Examples of this includes the ability of second-messenger molecules, including AC and PLC β , to act as RGS proteins, thus regulating their own activity, and the capacity of certain RGS proteins to generate or alter cellular signals. Another occurrence of novel function is the ability of signaling molecules downstream of one type of receptor to communicate with receptors/signaling molecules in a separate pathway. This crosstalk has been demonstrated to occur across ion channels and GPCR signaling cascades (G $\beta\gamma$ regulation of ion flux) and between RTKs and GPCRs (G α activation of MAPK pathway, ARF interacting with GPCRs), as illustrated in Fig 3.13. Such a detailed level of intracellular communication provides a mechanism for tight control of cellular processes and responses to external stimuli. Additionally, several proteins have been determined to play a role in bringing second-messenger molecules together for novel downstream signals, including GRKs and RGS proteins, and much research is currently directed at determining the unique signals generated through interaction with these proteins. The dynamic interplay that exists between pathways adds heightened complexity and control to traditional signaling cascades and yields new insight into potential points of regulation within the cell for experimental manipulation and innovative therapeutic approaches.

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4

NEURONAL NICOTINIC RECEPTORS: ONE HUNDRED YEARS OF PROGRESS

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4.1	Brief History of nAChRs	108
4.2	Neuronal nAChRs: Overview	110
4.3	Structure of nAChRs	111
4.4	Pharmacology of Major Neuronal nAChR Subtypes	113
4.4.1	The $\alpha 7$ nAChR	114
4.4.2	Heteromeric nAChRs	116
4.4.2.1	Pharmacology of Simple Heteromeric nAChRs	118
4.4.2.2	Pharmacology of Mixed Heteromeric nAChRs	122
4.4.2.3	Distribution of Heteromeric nAChRs	124
4.5	Regulation of nAChRs	125
4.5.1	Desensitization and Inactivation of nAChRs	125
4.5.2	Agonist-Induced Up-regulation of nAChRs	127
4.6	Conclusions	130
	References	131

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that when activated open, allowing sodium and calcium ions to flow into the cell and potassium ions to flow out of the cell, down their concentration gradients. This leads to depolarization of the membrane and an excitatory cellular response. These receptors are absolutely fundamental to physiology if for no other reason than that they mediate the fast, nearly fail-safe neurotransmission provided by acetylcholine at skeletal muscle and at virtually all autonomic ganglia, the adrenal gland, and sensory ganglia. Through these functions they influence virtually all organ systems in the body.

In addition to the critical physiological functions that nAChRs serve in the peripheral nervous systems, they are also found throughout the central nervous system (CNS), where, beyond their roles in mediating actions of endogenous acetylcholine, they are the targets for nicotine, the addictive agent in tobacco and

the main reason that people smoke (or at least the main reason so many have difficulty giving up smoking). In North America, about 50 million people smoke cigarettes regularly and the number is estimated to be *more than a billion worldwide*. Cigarette smoking is considered to be the most important preventable cause of early morbidity and mortality in most industrialized countries [1]; and if it exacts a lesser toll in developing countries it is probably only because the ravages of other health problems, such as famine, drought, poor nutrition, malaria, and other infectious diseases are, for the time being, more effective agents of depredation and premature death.

But obviously, nAChRs in brain did not evolve to mediate the stimulatory and addictive effects of nicotine; in fact, these receptors are widely distributed throughout the CNS, and they are frequently found on neurons of several major neurotransmitter systems. These strategic locations allow nAChRs to influence the release of dopamine, norepinephrine, acetylcholine, γ -aminobutyric acid (GABA), and glutamate (for a concise review of neurotransmitter release mediated by nicotinic receptors, see [2]). These neurotransmitters are, of course, associated with diverse effects, including arousal, reward, mood/affect, appetitive behaviors, neuroendocrine functions, and cognition. Thus, considering their effects on the CNS and the autonomic nervous system, neuronal nAChRs would be expected to influence multiple aspects of behavior, physiology, and the human condition. This chapter will review some of the fundamental aspects of these receptors, with emphasis on the different subtypes of neuronal nAChRs, their pharmacology, and their unusual regulation. For a review of nicotine addiction, see Chapter 15 in Volume II of this handbook.

4.1 BRIEF HISTORY OF nAChRs

It might seem a little unusual to begin an account of the contemporary neuropharmacology of a neurotransmitter receptor with its history, but it may be instructive to glimpse into the early studies of these receptors, in the years well before the advent of molecular biology, cloning strategies, ligand binding methods, patch clamp recordings, or, for that matter, most of the modern techniques that we rely on so heavily and more or less take for granted. The nicotinic receptors were the first receptors to be systematically explored, and their study laid the foundation for much of receptor biology. In fact, it was during studies of the actions of nicotine and the South American arrow poison curare on muscle that John Langley coined the term “receptive substance” [3, 4] and thus, along with Paul Ehrlich, who was studying antimicrobials and antigen–antibody interactions [5], developed the concept that small molecules such as hormones, antibodies, and exogenous chemicals (drugs) acted at specialized molecules on biological tissues. The receptor concept and theory led directly to the quantitative analyses of the actions of hormones, neurotransmitters, and drugs. But for many decades following the publications by Langley and Ehrlich, receptors were accepted, if at all, more as a concept than as actual biological entities that one day might be known in great detail. That was, of course, because many of the tools necessary to ask critical questions about receptors and probe their nature had not yet been developed.

The wide acceptance of nAChRs as more than a concept changed in the 1960s and early 1970s, especially after the isolation of nAChRs from the electric organ of fish and the demonstration of their function by Changeux and colleagues [6]. There

followed a series of detailed analyses of the molecular nature of nicotinic receptors from the electric organ of *Torpedo*, which laid the foundation for much of the understanding of nicotinic receptors at a molecular level three decades later (for reviews see [7–10]). Moreover, even though the receptors from electric organs are more similar to the mammalian muscle nAChR, their isolation and study led directly to the cloning of the neuronal nAChRs in mammalian brain and ganglia. The nAChR is now considered the prototype for the “Cys-loop” superfamily of ligand-gated ion channels, which includes GABA and glycine receptors as well as serotonin-3 receptors.

Our understanding of nAChRs has been greatly advanced by the clever use of certain natural products—indeed, probably no other biological molecule has been studied with so many unusual gifts of nature. The first recorded description by Europeans of the use of curare-tipped arrows by people of South America dates to the fifteenth century, but it is safe to say that the indigenous populations had used curare for its muscle-paralyzing actions in hunting and in defense long before the Europeans happened by (see [11] for an interesting account of early descriptions of curare use as related in the writings of fifteenth-century Spanish explorers). In the mid-nineteenth century, curare, which was isolated from crude plant sources, became an important tool in studies of muscle physiology. Notably, Claude Bernard used preparations of curare to block nicotine’s action when applied at the junction of nerve and muscle. Fifty years later, Langley would use these same two natural compounds to support the formulation of the receptor concept.

We move next to the 1937 World’s Fair in Paris, where David Nachmansohn, a biochemist studying nerve conduction and muscle contraction, chanced upon a demonstration of the power of an electric fish (*Torpedo marmorata*). Nachmansohn was interested in the bioenergetics of acetylcholine and acetylcholinesterase, the enzyme that hydrolyzes it, and he had recently learned that the fish electric organ was a kind of modified muscle. He began a series of studies that demonstrated that electric tissue of fish was extremely rich in acetylcholine and acetylcholinesterase, and he established that acetylcholine was in fact responsible for generating the electric charge (for a retrospective of the earliest studies of the cholinergic parameters of *Torpedo* electric tissue, see [12]).

The discovery of acetylcholine’s critical role in fish electric organs led 30 years later to the studies by Changeux and colleagues that resulted in the isolation of the prototypical nAChR from electric tissues; but these innovative studies required yet another critical gift from nature, this time from a snake. The venom of the Taiwanese banded krait, *Bungarus multicinctus*, contains a powerful toxin that paralyzes muscle. Chang and Lee [13] had isolated this protein and determined that it acted by potently and nearly irreversibly blocking the nicotinic receptors at the muscle end plate. They named it α -bungarotoxin, based on its position after electrophoretic separation. As related by Chang [14] in a brief retrospective, the isolation of α -bungarotoxin and elucidation of its mode of action did not attract much attention until Lee visited Changeux in Paris and provided him with a sample. Changeux, Lee, and their colleagues used the toxin to probe the nAChR in electric tissue [6] and eventually to purify the receptor [15].

Early studies of the nAChR purified from electric tissue revealed critical information about it, including the number of subunits in the receptor (four: α , β , γ , δ , with the α subunit represented twice, accounting for a pentameric structure), their partial

amino acid sequences, the flow of cations through the channel formed by the subunits, and the location of the acetylcholine binding site on the α subunits (reviewed in [7, 8, 16, 17]). The receptor has been imaged at 9 Å resolution in three-dimensional reconstructions from electron microscopy images [18] and more recently at 4 Å resolution [19]. Thus two molecules—proteins that for millions of years have provided their owners with means of survival—were brought together for the purpose of illuminating a fundamental question in biology and in fact contributed to the revolution in neurobiology that is still taking place. (It is unlikely that either the electric fish or the banded krait is aware of their critical roles in this.)

Beginning in 1982, several laboratories used cloning strategies to deduce the amino acid sequences of the α and γ subunits of the *Torpedo* electroplax receptor from their cDNA sequences [20–25]. And soon after, Numa and colleagues deduced the full amino sequences of all four subunits (α , β , γ , δ) from electroplax receptors [26, 27] as well as that for the α subunit of calf and human muscle ([28]; see [29] for a review). The amino acid sequences of the four subunits of electroplax are 40–65% identical, which suggests that they are the products of gene duplications over time. Moreover, the subunits seem to have been very good at what they do because they have been highly conserved across >400 million years of evolution, so that, for example, the 437 amino acids in the α subunit of *Torpedo* electric organ and human muscle differ by only $\sim 20\%$ [28, 30]. Knowing the sequences for the receptor subunits in electroplax provided a path toward understanding the neuronal nicotinic receptors. But there have been many surprises along the way.

4.2 NEURONAL nAChRs: OVERVIEW

The role of acetylcholine in autonomic ganglia neurotransmission has been known for most of the twentieth century (for an early review see [31]); and since the responses of ganglia to cholinergic nerve stimulation can be mimicked by application of nicotine, the receptors that mediate these responses are classified as nicotinic cholinergic, like the skeletal muscle. But early on it was recognized that certain drugs that potently block transmission at the neuromuscular junction are much less potent or even ineffective in blocking ganglionic transmission and vice versa. For example, it was noted that in a series of bis-methonium compounds containing identical quaternary nitrogen groups at each end but separated by a variable number of simple methylene groups, that is, $(-\text{CH}_2-)_n$, the number of methylene groups determines whether a compound is a potent blocker of ganglia or of muscle [32]. Hexamethonium, the compound containing six methylene groups, effectively blocked ganglia, while decamethonium effectively blocked muscle. Since the only difference between these two compounds is the number of carbon atoms in the methylene chain that separates the two positively charged methonium head groups, the difference in potency was thought to reflect the topology of the receptors in the ganglia and muscle, specifically the distances between anionic groups on the receptor that could accommodate the two cationic groups of the blocking drugs [33]. It is now thought that while the distance between the anionic groups is one determinant, it is not the only one.

Despite these differences, the genes coding for the receptors in the nervous system proved to be similar enough to the muscle-type receptor genes to allow hybridization to the muscle subunit cDNA probes under low-stringency conditions. Thus, having

the cDNA for the subunits of the electrophysiological and mammalian muscle receptor allowed the isolation and cloning of multiple receptor subunit genes in the nervous system. That initial strategy was well rewarded, and since the isolation of the subunits from neuronal tissues and the location of their mRNA in rat brain [34–39], nine different α subunits ($\alpha 2$ – $\alpha 10$) and three different β subunits ($\beta 2$ – $\beta 4$), all distinct from the muscle receptor subunits, have been found in vertebrate neuronal tissues (for several excellent and comprehensive reviews on the diversity of the neuronal nicotinic receptors, see [40–43]).

4.3 STRUCTURE OF nAChRs

Many of the salient structural characteristics of nAChRs have been derived from years of elegant studies of vertebrate receptors (for several excellent detailed reviews see [9, 19, 42, 44]), and recently our understanding of many of these structural features has been reinforced and some new ones revealed by X-ray crystallography of a molluscan acetylcholine binding protein that resembles the amino terminal of the $\alpha 7$ subunit [4, 5].

All subunits consist of a polypeptide chain with a relatively large hydrophilic amino terminal followed by four hydrophobic transmembrane domains, designated M1–M4, and a relatively short C-terminus (Fig. 4.1). A large intracellular loop formed between M3 and M4 in all of the subunits contains the most variable sequences. The extracellular N-terminus contains a disulfide bond formed by two cysteines separated by 13 amino acids. This cys loop is common to all nAChR subunits, as well as to the subunits of the GABA_A, GABA_C, glycine, and serotonin-3 receptors, giving this receptor superfamily its name. Like the muscle receptor, the neuronal nAChRs are composed of five subunits assembled around a central ion channel so that their M2 transmembrane domains and a portion of the M1 domains line the channel (Fig. 4.1b) and contribute to the ion gating mechanism and probably to the state of the channel. Different combinations of the 12 α and β neuronal subunits comprise the pentameric receptors (Fig. 4.1c) and define its subtype.

The amino acid sequences of the subunits designated as $\alpha 2$ – $\alpha 10$ all have ~50% homology with the muscle $\alpha 1$ subunit overall, but the homology is much greater in the membrane-spanning regions [40]. All neuronal α subunits have in common with the $\alpha 1$ subunit of muscle a second disulfide bond formed from adjacent cysteine amino acids at or near positions 192–193 in the sequence. This disulfide bond exerts a powerful influence on the binding site for acetylcholine and other agonists [46–49], and in fact it is a major criterion for belonging to the α -subunit class. There is less sequence homology between the neuronal $\beta 2$ – $\beta 4$ subunits and the $\beta 1$ subunit of muscle or even among the neuronal β subunits themselves [40, 41]. The acetylcholine binding site is formed at the interface of an α subunit and an adjacent subunit; however, as will be described below, not all α and β subunits are equal in this regard.

When certain combinations of α and β subunits are expressed in heterologous systems, such as *Xenopus* oocytes or mammalian cell lines, they can form nAChR agonist binding sites and functional channels that represent potential nAChR subtypes. Examples of generic nAChR subtypes are shown in Figure 4.1c. Although all of the subtypes conduct Na⁺, K⁺, and Ca²⁺, their channel and pharmacological properties can vary considerably, as does their regulation during exposure to

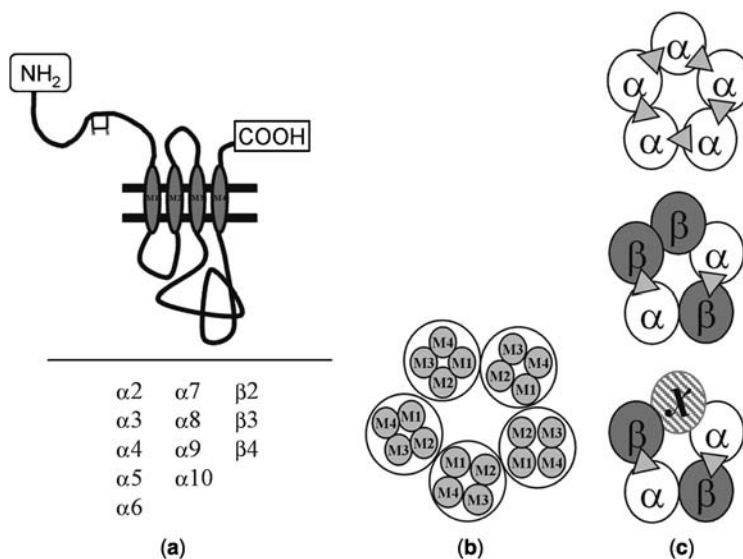


Figure 4.1 Basic structure of neuronal nAChRs. (a) Neuronal nAChRs are pentameric proteins composed of five subunits from among nine α and three β subunits. Each subunit consists of a large N-terminus of ~ 210 amino acids, four transmembrane domains designated M1–M4, and a short C-terminus. A large cytoplasmic loop forms between M3 and M4 of all subunits and contains the most variable sequences among the different subunits. The extracellular amino terminal contains a disulfide bond formed by two cysteines separated by 13 amino acids. This Cys-loop is common to all nAChR subunits, as well as to the GABA_A, GABA_C, glycine, and serotonin-3 receptors. A second disulfide bond, formed from adjacent cysteine residues at about positions 192–193, close to the first transmembrane segment, is common to all α subunits and exerts a strong influence on the agonist binding site. (b) The M2 and a portion of the M1 transmembrane segments of each subunit line the channel and contribute to its ion selectivity. In addition, the different states of the channel—open, closed, desensitized—are probably executed here by an allosteric mechanism. (c) Neuronal nAChR subtypes can be homomeric receptors, comprised of $\alpha 7$, $\alpha 8$ or $\alpha 9$ subunits only; simple heteromeric receptors, comprised of combinations of one type of α and one type of β subunit; or *mixed* heteromeric receptors, comprised of one or more types of α subunit combined with one or more types of β subunit.

nicotine. Most of the known nAChR subtypes are heteromeric—that is, they are composed of combinations of α and β subunits. Simple heteromeric receptors are formed from a single type of α subunit combined with a single type of β subunit—for example, $(\alpha 4)_2 (\beta 2)_3$. *Mixed* heteromeric receptors contain one or more types of α subunit combined with two or more types of β subunit, or vice versa. Some of the nAChRs in chick ciliary ganglia are mixed heteromeric receptors composed of $\alpha 3$, $\alpha 5$ and $\beta 4$ subunits, presumably $(\alpha 3)_2 (\alpha 5)(\beta 4)_2$ [50, 51], and several different mixed heteromeric subtypes have been found in some CNS regions from chick [52, 53] and rat [54–57], which in some cases contain receptors with up to four different subunits.

The $\alpha 7$, $\alpha 8$, and the $\alpha 9$ subunits can form homomeric receptors composed of five identical α subunits without the need for a β subunit. In particular, the $\alpha 7$ subunit forms a homomeric receptor that is highly represented in the CNS and has been implicated in a variety of synaptic and extra synaptic functions. It is the most widely

distributed and the most widely studied of the homomeric receptors (for reviews see [58, 59]. The $\alpha 8$ subunit has so far been found only in avian species. The $\alpha 9$ subunit has a very limited distribution, but it appears to associate with α_{10} subunits to form a nAChR in cochlear hair cells [60]. Interestingly, these receptors have recently been implicated in the ototoxicity that has long been associated with the antimalarial compounds quinine quinidine, and chloroquine [61].

The rules of assembly for the α and β subunits are not completely known; therefore, the number of receptor subtypes that actually exist in the vertebrate nervous system is not known. However, a few important “rules” have emerged from studies of subunit combinations expressed heterologously in oocytes or in mammalian cells. These rules are helpful in thinking about the potential diversity of the neuronal nAChRs in native tissues and in assigning subtypes to particular functions or locations. Thus, the rules of assembly established so far are as follows:

1. The $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 6$ subunits require association with either $\beta 2$ or $\beta 4$ subunits to form functional ligand-gated ion channels, and, based on the muscle receptor stoichiometry, the neuronal receptors are thought to contain at least two α subunits.
2. The $\alpha 5$ subunit requires association with other α subunits as well as $\beta 2$ or $\beta 4$ subunits to form a functional receptor or agonist binding site [62, 63]. Similarly, the $\beta 3$ subunit requires both other β subunits as well as other α subunits to form a receptor [64]. Thus, the presence of either the $\alpha 5$ or the $\beta 3$ subunit indicates that a receptor is a mixed heteromeric subtype.
3. As stated above, the $\alpha 7$, $\alpha 8$, and $\alpha 9$ subunits can each form homomeric receptors, and $\alpha 9$ can also associate with α_{10} to form a nAChR with unusual properties [60].

The full subunit composition of neuronal nAChR subtypes in most tissues is not yet known; therefore, when a receptor subtype in a tissue is tentatively identified by its pharmacological or biophysical characteristics, the convention of adding an asterisk has been adopted to signify that it may have one or more additional subunits [65]. For example, $\alpha 3\beta 4^*$ would designate receptors that are composed of $\alpha 3$ and $\beta 4$ subunits, but one or more other subunits might also be incorporated in at least some of the receptors, for example, $\alpha 3\beta 4\alpha 5$.

4.4 PHARMACOLOGY OF MAJOR NEURONAL nAChR SUBTYPES

As described above, the physiology and to some extent the pharmacology of nAChRs in ganglia and adrenal gland have long been recognized and studied. In fact, several early antihypertensive drugs, such as hexamethonium and mecamylamine, were aimed at nAChRs in autonomic ganglia. These nAChR blockers lowered blood pressure (sometimes quite dramatically) by blocking the signals at the ganglia that control blood vessel tone via postganglionic sympathetic axons that release norepinephrine; but because these drugs block signals at *all* autonomic ganglia, they also produce a wide range of unacceptable but entirely predictable side effects. Since

there are now many drugs that lower blood pressure via several more subtle and scalable mechanisms, ganglionic nAChR blockers are seldom if ever used clinically.

It is curious that, except for the recognition that the recurrent collateral inhibition of spinal motor neurons involves nAChRs on the inhibitory Renshaw interneuron, there seems to have been very limited exploration of the role of nAChRs in the CNS until the early 1970s. This despite the fact that during a large part of the twentieth century a significant fraction (up to 50%) of the adult populations of the United States, Canada, and most European nations smoked cigarettes. The biological underpinnings for this behavior, even in the face of the well-known health risks of smoking, was a subject of some conjecture. One intriguing finding, however, was that nicotine could release dopamine and norepinephrine from rat brain slices [66–69]; and the possible connection between these catecholamines and other stimulant drugs was already widely suspected [70].

4.4.1 The $\alpha 7$ nAChR

Studies in the 1970s established the existence of binding sites for [125 I] α -bungarotoxin ([125 I] α -BTx) in mammalian brain. These sites were at first thought to be similar to the muscle nAChR, but we now know these receptors as the homomeric $\alpha 7$ nAChR subtype, which are structurally quite different from the muscle receptor. Because it is essentially the only nAChR in mammalian brain that binds α -BTx with high affinity, the homomeric $\alpha 7$ receptor can be located and measured definitively in binding studies [49, 71, 72]. Interestingly, early studies found that α -BTx, which you will recall blocks the muscle nAChRs very potently and nearly irreversibly, did not block responses evoked by acetylcholine or nicotine in some neuronal cells, such as PC12 cells (a rat neuroendocrine cell) and rat and chick sympathetic ganglia [73–75].

The failure of α -BTx to block acetylcholine-stimulated responses in neuronal tissues led to some early speculation that the [125 I] α -BTx binding site might not represent a nicotinic cholinergic receptor. Moreover, because homomeric $\alpha 7$ nAChRs desensitize rapidly upon application of agonists, they were difficult to measure with earlier patch clamp techniques. But since then, the introduction of fast application methods for patch clamp recording has allowed extensive studies of these receptors in heterologous expression systems as well as in neuronal tissues. The $\alpha 7$ receptor is notable for its large calcium conductance, which equals that of N-methyl-D-aspartate (NMDA) receptors [76, 77]. In part because they are often found outside the zones of synaptic specialties and because of their high calcium permeability, the $\alpha 7$ receptors have been implicated in a variety of neuronal functions, including “perisynaptic” as well as postsynaptic and presynaptic mediation of cholinergic signals (see [59] for a review).

Measurements of $\alpha 7$ receptors in mammalian brain can be made definitively with [125 I] α -BTx, and the pharmacology of the $\alpha 7$ receptor binding site in rat and mouse brain has been well studied [49]. However, there is sometimes significant disparity in the affinities reported for α -BTx in different studies, which is probably related to the different assay systems used in the studies (e.g., brain vs. heterologous expression systems) and the different methods used by various laboratories. In addition, in some cases the difference might derive partly from uncertainty of the specific radioactivity of the [125 I] α -BTx used and consequent uncertainty in the actual concentration of the radioligand used in the competition studies. Thus two- to five-fold differences in K_i

values derived from a logscale for the same drug are not uncommon. Nevertheless, there is good agreement on the rank order of drug affinities measured in different laboratories. Table 4.1 compares the K_i values for several drugs competing with [125 I] α -BTx for binding at $\alpha 7$ receptors measured in rat brain membranes with values measured at human $\alpha 7$ receptors heterologously expressed in SH-EP1 cells (a human epithelial cell line). The rank order and even the K_i values measured in the two studies are in general agreement, especially considering again that the measurements are from different laboratories derived with somewhat different methods. However, the difference in binding values for α -BTx itself is notable, so it will be important to learn if the difference in affinity of α -BTx accurately reflects an actual difference between the rat and human receptor or is related to differences in the processing of the receptors in native tissues and the heterologous expression system.

In measurements of function by whole-cell patch clamp recordings, acetylcholine, nicotine, and cytisine are full agonists in both chick $\alpha 7$ receptors expressed in oocytes [78, 79] and human $\alpha 7$ receptors expressed in mammalian cells [80, 81]. Interestingly, choline, the precursor to acetylcholine in its synthesis as well as a product of acetylcholine hydrolysis after its release, is an agonist at $\alpha 7$ receptors [81, 82]. As is the case for virtually all nAChRs, the potencies of agonists to activate $\alpha 7$ receptor channel function is significantly lower than their affinities measured in binding assays would have predicted (i.e., agonist EC_{50} values are much higher than their K_i values). This reflects the difference in the receptor conformation in its resting state, from which it can be activated, and its desensitized state, which binds agonists with much higher affinity but is essentially nonactivatable. Interestingly, 1,1-dimethyl-4-phenylpiperazinium (DMPP) appeared to be a very weak agonist ($\sim 5\%$ compared to nicotine) at the chick $\alpha 7$ receptor expressed in frog oocytes [78, 79] but nearly a full agonist at human $\alpha 7$ receptors expressed in mammalian cells [80]. Again this could be due to the different origins of the receptor or the cell type in which it was expressed.

TABLE 4.1 K_i Values of Drugs Binding at $\alpha 7$ nAChRs from Rat Brain and at Human $\alpha 7$ nAChRs Expressed in SH-EP1 Cells

Drug	K_i (nM)	
	Rat Brain $\alpha 7$	Human $\alpha 7$ Cells
Agonists		
Acetylcholine	4,000	—
Nicotine	820	420
Cytisine	1,400	380
Carbamylcholine	12,000	3,500
DMPP	470	40
Antagonists		
<i>d</i> -Tubocurarine	2,800	3,000
α -BTx	0.16	7
Methyllycaconitine	1.1	14

Note: Data for rat $\alpha 7$ receptor from [49], except methyllycaconitine, which are from [77a]. Data for human $\alpha 7$ receptor from [77b].

4.4.2 Heteromeric nAChRs

The suspicion early on that the [125 I] α -BTx binding sites might not represent the only neuronal nAChR led to a search for other brain nicotinic binding sites with characteristics that could be related to nAChRs. In 1979 and 1980, three papers reported [3 H]nicotine binding sites in brain [83–85]. These sites had many of the pharmacological characteristics expected of a nicotinic receptor, but α -BTx did not compete effectively for them. A short time later studies using [3 H]acetylcholine [86] and [3 H]nicotine [87] added further evidence that that these binding sites in brain probably represented the agonist recognition sites of a neuronal nAChR. Moreover, not only was α -BTx an ineffective competitor for the binding sites labeled by these agonists but the brain distributions of the high-affinity agonist binding sites and the α -BTx binding sites were markedly different, indicating that they were not even part of the same receptor molecule [72, 86, 87]. These studies were thus a strong indication that there is more than one type of nAChR in brain. Interestingly, the studies with the agonist radioligands found that several effective classical antagonists of ganglionic nAChRs, such as mecamylamine and hexamethonium, do not compete effectively for [3 H]acetylcholine and [3 H]nicotine binding sites. That is because these and several other ganglionic antagonists act primarily by blocking the receptor channel, rather than by competing for the agonist binding site [88, 89].

Later studies established that the brain binding site for the [3 H]-agonist ligands, as well as that for [125 I] α -BTx, depended on an intact disulfide bond that could be reduced and reoxidized with corresponding loss and restoration of agonist binding [48, 49]. This requirement for an intact disulfide bond in brain nAChR binding sites is similar to that in electroplax and muscle nAChRs [46, 47], and in fact, it appears to be a common feature of the agonist recognition site of all nAChRs.

[3 H]Acetylcholine and [3 H](–)nicotine label the same receptor sites in rat and mouse brain [49, 90]; importantly, however, neither is an effective ligand for labeling and measuring neuronal nAChRs in autonomic ganglia, adrenal gland, or PC12 cells, all of which are known to contain nAChRs—indeed, these receptors mediate the primary functions of autonomic ganglia and adrenal chromaffin cells. In the early 1990s, studies with [3 H]cytisine established it as an excellent radioligand for measuring nAChRs in brain [91–94]. It has 10–20 times higher affinity than [3 H]acetylcholine or [3 H](–)nicotine and low nonspecific binding (which translates into a better signal-to-noise ratio). But even this ligand was ineffective for measuring neuronal nAChRs in ganglia and PC12 cells. The reasons that these radioligands were unable to measure neuronal nAChR binding sites in ganglia and PC12 cells became apparent when the pharmacology of the predominant receptor subtypes in these tissues was better understood and the narrow spectrum of all of these radioligands was realized.

Rat brain expresses the genes for nearly all of the nAChR α and β subunits [40, 95]. The mRNA transcripts have different distributions, but there is extensive overlap among some of them. For example, the $\alpha 4$ and $\beta 2$ subunit mRNAs overlap in most brain regions [37] and the $\alpha 3$ and $\beta 4$ subunit mRNAs overlap in many brain areas [96]. There is sometimes a significant mismatch between the location of subunit mRNA and its cognate protein. There may be several reasons for this mismatch, but one important factor is that neuronal nAChRs are often found on axons and axon terminals some distance from the cell bodies, where mRNA is typically concentrated. Fortunately, the development of nAChR subunit-selective antibodies has provided

critical probes to measure the specific subunits in the nervous system ([97–103]; for a review, see [104]). Moreover, these antibodies have provided tools to determine the subunit composition of different heteromeric neuronal nAChR subtypes and have thus allowed major subtypes, defined by their subunit composition, to be identified in several areas of the nervous system. For example, using antibodies, the predominant nAChR in rat and chick forebrain was determined to be the $\alpha 4\beta 2$ subtype [52, 105, 106]. In contrast, in the ganglia that have been studied the $\alpha 4\beta 2$ subtype exists at low levels, if at all, and the major, though not the only, nAChR appears to be an $\alpha 3\beta 4^*$ subtype [62, 107, 108].

Studies of nAChR activation in oocyte expression systems had established clear differences in the pharmacology of receptors that contain $\beta 2$ versus $\beta 4$ subunits [109–111], but the inability to radiolabel and measure nAChRs in ganglia was perplexing, and, more important, it was a significant obstacle to delineating the pharmacology of the diverse members of the neuronal nAChR family and exploring their roles and regulation. Moreover, it was apparent that if the [^3H] agonists in use at the time did not label the receptor subtype(s) in ganglia and PC12 cells, they probably did not label these subtypes if they existed in the CNS either.

The solution to this problem came in the form of yet another gift from nature, the discovery of which is itself a fascinating story. As detailed by Daly [112, 113] and summarized here, in the mid-1970s Daly and colleagues extracted an alkaloid from the skin of a small Ecuadoran poison dart frog, *Epipedobates tricolor*. When injected into mice the extract elicited a Straub-tail response, which is usually indicative of opioid-like activity. Thus the alkaloid was of interest as a possible analgesic. Indeed, further studies with about 500 μg of the purified alkaloid isolated by column chromatography showed it to be ~ 200 times more potent than morphine in preclinical tests of analgesic activity. But surprisingly, the analgesic activity was not blocked by naloxone, a potent opioid antagonist. Clearly this alkaloid was potentially very important and of great interest. Unfortunately, in the years between the alkaloid's initial discovery by Daly and colleagues and its subsequent isolation several years later, the source of this important and mysterious alkaloid all but disappeared. Studies on extracts from *E. tricolor* subsequently collected in Ecuador showed they had very low levels of the active alkaloid, and specimens of *E. tricolor* that had been brought out of Ecuador and raised in the United States did not produce any of the material. It is now thought that the alkaloid of interest was a product of the frogs' diet in their indigenous habitat. Daly waited until 1990, when he thought that the necessary analytical techniques had become sensitive enough to solve the structure of the alkaloid from the small amount of the original sample he had wisely held back. The gamble with the world's entire supply of the active material worked and the structure of epibatidine, the active alkaloid, was published in 1992 [114]. Within two years of the publication of its structure, no fewer than eight laboratories published synthesis schemes [115].

Studies with the new supplies of synthesized epibatidine revealed more remarkable features. Epibatidine's potent analgesic activity in mice was blocked by the noncompetitive nicotinic antagonist mecamylamine [112, 113], indicating that it is an agonist. Moreover, it displayed very high affinity ($\sim 50\text{ pM}$) for heteromeric neuronal nAChRs labeled by [^3H](–)nicotine in rat brain. The high affinity of epibatidine in binding studies paralleled its agonist potency in functional studies, although like all nicotinic agonists that have been studied, its affinity for its receptors

as measured by its dissociation constant (K_i or K_d) in binding studies is 50 to 300 times higher than its potency (EC_{50}) in functional studies.

In 1994, [3H]epibatidine ([3H]EB) was developed as a ligand to measure nAChRs, and studies with it quickly revealed that it labeled not only the heteromeric nAChRs in brain but also those in adrenal gland and retina; furthermore, it displayed very low nonspecific binding over a wide concentration range [116–118]. The affinities of [3H]EB for its binding sites in brain and ganglionic tissues were found to be ~ 50 and 300 pM, respectively, a hint of the marked differences in the pharmacology of the receptors in these two tissues. Epibatidine, which contains a chlorine atom in its ring structure, can also be synthesized with an iodine and still retain high affinity for nAChRs [112]. [^{125}I]EB has since been synthesized [119] and has proven to be particularly useful for measuring nAChRs in tissues that are in limited supply or with a low density of receptors, and especially for mapping nAChRs in autoradiography studies, where exposure times can be decreased from the several weeks often required with [3H]EB to one to four days [120, 121].

4.4.2.1 Pharmacology of Simple Heteromeric nAChRs. Ligand binding studies with [3H]EB have helped to elucidate the pharmacology of the diverse subtypes of nAChRs in the nervous system. For example, [3H]EB binds with high affinity to each of six different simple heteromeric rat nAChR subunit combinations expressed in oocytes [122] and in mammalian cells [123]. This has allowed the pharmacological profiles of the binding sites for these possible receptor subtypes to be clearly delineated. Table 4.2 shows the pharmacology of these binding sites expressed in the mammalian cells, but the pharmacological profiles of the receptors expressed in oocytes are similar. These comparative studies across subunit combinations showed that, in general, all of the nicotinic agonist drugs examined have higher affinity for combinations formed with the $\beta 2$ subunit than with the $\beta 4$ subunit. In particular, acetylcholine, nicotine, and cytosine, all of which were among the earliest radiolabeled ligands for nAChRs, display a dissociation constant (K_i) for the $\alpha 3\beta 4$ subunit combination of 200 nM or greater. This translates into an affinity that is far too low to reliably radiolabel receptors comprised of this subunit combination, which explains why these ligands are not very useful as radioligands for the nAChRs in ganglia.

A-85380, a pyridyl ether compound synthesized and characterized at Abbott Laboratories [124, 125], discriminates particularly well between nAChRs containing $\beta 2$ and $\beta 4$ subunits. In fact, it and its iodinated analog, 5-iodo-A-85380, display 50- to >1000 -fold higher affinities at the $\beta 2$ -containing subunit pairs than the $\beta 4$ -containing pairs (Table 4.2). Importantly, this selectivity extends to comparisons between the $\alpha 4\beta 2$ subunit combination, which represents the predominant receptor in brain, and the $\alpha 3\beta 4$ combination, which represents the predominant receptor in many autonomic ganglia.

Earlier studies measuring current responses in oocytes expressing α and β subunit pairs demonstrated that both subunits influence the sensitivity of nAChRs to agonist drugs [109]; and although the β subunit seems to exert the major effect on the binding site pharmacology, the α subunit also clearly influences the binding site pharmacology. For example, several agonists have a slightly higher affinity for the $\alpha 4\beta 2$ than for the $\alpha 3\beta 2$ subunit combination; but cytosine in particular displays a marked difference, which has helped to tentatively identify the relatively few populations of $\alpha 3\beta 2^*$ receptors in the rodent brain [121, 126]. Similarly, the much higher affinity of A-85380

TABLE 4.2 Comparison of K_i Values of Nicotinic Drugs at Six Different Rat nAChR Subunit Combinations Stably Expressed in Transfected Mammalian Cells

Ligand	K_i (nM)					
	$\alpha 2\beta 2$	$\alpha 2\beta 4$	$\alpha 3\beta 2$	$\alpha 3\beta 4$	$\alpha 4\beta 2$	$\alpha 4\beta 4$
(\pm)-Epibatidine	0.025	0.095	0.035	0.57	0.061	0.16
(\pm)-I-Epibatidine	0.11	0.15	0.18	0.98	0.15	0.15
Acetylcholine	11	110	56	850	44	99
($-$) – Nicotine	12	110	47	440	10	40
Cytisine	1.1	5.4	37	220	1.5	2.1
A-85380	0.073	18	0.21	78	0.14	8.1
I-A-85380	0.031	41	0.47	280	0.059	24
Carbachol	260	930	1,100	4700	590	1,100
DMPP	32	1,400	35	820	82	2,700
Choline	14,000	26,000	49,000	58,000	35,000	45,000
DH β E	6,200	190,000	3,800	110,000	600	17,000
MLA	52,000	110,000	1,100	2,200	28,000	6,400

Source: Adapted from [123].

for $\beta 2$ -containing nAChRs compared to $\beta 4$ -containing nAChRs makes it useful as a pharmacological mask of the $\beta 2$ receptors, which has allowed the location and relative density of several clusters of $\beta 4$ -containing receptors (probably $\alpha 3\beta 4^*$ receptors) labeled by [125 I]EB in rodent brain to be mapped [121, 127] (see below).

As with any model system, the pharmacological profiles of these subunit combinations in heterologous systems are useful only to the extent that they reflect native nAChRs. Table 4.3 compares the binding affinities of drugs at heterologously expressed subunit combinations with native receptors in rat forebrain, which are predominantly the $\alpha 4\beta 2$ subtype, and pineal gland, which expresses the $\alpha 3\beta 4$ subtype virtually exclusively [128]. The close agreement between the values for the heterologously expressed subunit combinations and the native nAChRs from rat tissues provides a measure of confidence that the pharmacology of the binding sites in heterologous expression systems reliably represents the pharmacology of the binding site of the native receptors. Interestingly, the affinities of many of these drugs at the rat $\alpha 4\beta 2$ subunit combination appear to be lower than those at the human $\alpha 4\beta 2$ subunit combination expressed in SH-EP1 cells [129], but both the rank order and the relative affinities of the drugs in the two systems are very similar.

Activation of nAChR in heterologous expression systems has been studied in several ways. The most widely used method is whole-cell patch clamp recordings of subunit combinations expressed in oocytes [109, 110, 130, 131] or in mammalian cell lines [132–137]. In addition to whole-cell recordings in mammalian cells, nAChR function has been assessed using measurements of $^{86}\text{Rb}^+$ efflux through the receptor channel [129, 138–140] and measurements of fluorescence linked to changes in membrane potential and/or intracellular calcium [141, 142]. Whole-cell patch clamp recordings are very sensitive and provide real-time, direct measurements of physiological responses, while ion flux and fluorescence-based assays allow relatively rapid comparisons across nAChR subunit combinations and among drugs.

TABLE 4.3 K_i Values for Drugs Measured in Native nAChRs and Heterologous $\alpha 4\beta 2$ and $\alpha 3\beta 4$ Receptors Expressed in HEK cells

Drug	K_i (nM)			
	Rat $\alpha 4\beta 2$ Subunits	Rat Forebrain ^a	Rat $\alpha 3\beta 4$ Subunits	Rat Pineal Gland ^a
Epibatidine	0.016	0.059		
Acetylcholine	44	45		
Nicotine	10	12	318	202
Cytisine	1.5	1.9	128	63
A-85380	0.14	0.25	54	24
I-A85380	0.059	0.11		
Carbamylcholine	590	460		
DMPP	82	100		
DH β E ^a	600	130	99,000	65,000

^aNote: The native receptors in the rat forebrain are predominantly an $\alpha 4\beta 2$ subtype and those in rat pineal gland are nearly exclusively the $\alpha 3\beta 4$ subtype.

Data for $\alpha 4\beta 2$ subunits and rat forebrain adapted from [123]; data for $\alpha 3\beta 4$ subunits and pineal gland from [128].

Considering the different expression systems and measurements that have been used to assess the pharmacology of functional responses, it is not surprising that there is less agreement in the EC_{50} values than in the binding site K_i values for drugs across various nAChR subunit combinations, or even at any one subunit combination. There is, however, agreement on several important points:

1. Most nicotinic agonists are more potent at heterologously expressed $\alpha 4\beta 2$ nAChRs than at $\alpha 3\beta 4$ receptors. An example of this can be seen in Table 4.4, which compares agonist-stimulated $^{86}\text{Rb}^+$ efflux in cells expressing the human $\alpha 4\beta 2$ receptor [129] and the rat $\alpha 3\beta 4$ receptor [140]. The difference in potency at these two receptor subtypes parallels the higher binding affinity of agonists for the $\alpha 4\beta 2$ receptors.
2. Cytisine is a weak partial agonist at $\beta 2$ -containing receptors, eliciting only 10%–40% of the response stimulated by acetylcholine or nicotine; in contrast, it is nearly a full agonist at $\alpha 3\beta 4$ receptors. The low efficacy of cytisine at $\beta 2$ containing nAChRs was first noted in oocyte studies [109, 110] and has also been a consistent finding in $\alpha 4\beta 2$ receptors expressed in mammalian cells. It is an important feature that can help to determine whether a nicotinic response in native tissues is mediated by a nAChR containing $\beta 2$ or $\beta 4$ subunits.
3. DMPP usually appears to be a partial agonist with $\sim 70\%$ efficacy at the heterologously expressed $\alpha 4\beta 2$ receptor subtype [129, 138] as well as at the $\alpha 3\beta 4$ receptor subtype [140].
4. Epibatidine is the most potent agonist currently known. Furthermore, it sometimes appears to have greater efficacy than acetylcholine or nicotine. This greater efficacy probably results from its ability to elicit a full agonist

TABLE 4.4 Comparison of EC₅₀ Values of Nicotinic Agonists to Stimulate ⁸⁶Rb⁺ Efflux Through Human $\alpha 4\beta 2$ nAChRs Expressed in SH-EP1 Cells [129] and Rat $\alpha 3\beta 4$ nAChRs Expressed in HEK293 Cells [140]

Drug	EC ₅₀ (μ M)	
	Human $\alpha 4\beta 2$	Rat $\alpha 3\beta 4$
Epibatidine	0.0085	0.06
Nicotine	0.85	31
Cytisine	1.3	24
Acetylcholine	1.7	110
DMPP	1.9	28

response at concentrations well below those that begin to block the receptor channel, whereas, in contrast, less potent agonists appear to produce some channel blockade even within the concentration range necessary to measure their full activation of receptors.

5. Mecamylamine and hexamethonium, classic ganglionic blockers, appear to act noncompetitively by blocking the nAChR channel, rather than by competing for the agonist recognition site.
6. Dihydro- β -erythroidine (DH β E), in contrast to the classic ganglionic blockers, is a competitive antagonist, and like agonists, it is more potent at $\alpha 4\beta 2$ receptors than at $\alpha 3\beta 4$ receptors.

Again, because essentially all of the nAChRs in the rat pineal gland are the $\alpha 3\beta 4$ subtype, it has provided a good tissue in which to compare the functional responses of native and heterologously expressed receptors. As shown in Table 4.5, the EC₅₀ values for nicotinic drugs to activate whole-cell currents via $\alpha 3\beta 4$ nAChRs in rat pineal cells in primary culture are very similar to the values to activate ⁸⁶Rb⁺ efflux through $\alpha 3\beta 4$ nAChRs heterologously expressed in HEK293 cells. Thus, despite the very different methods used to assess receptor-mediated responses, the potency of a drug to activate the heterologously expressed $\alpha 3\beta 4$ receptors provides an accurate measurement of its potency at native receptors. Moreover, DMPP, which is a partial agonist at heterologously expressed $\alpha 3\beta 4$ receptors, is also a partial agonist at the $\alpha 3\beta 4$ receptors in the rat pineal gland [128].

Comparisons between native and heterologously expressed receptor subtypes are limited because there are so few native tissues that contain a preponderance of any one nAChR subtype. The $\alpha 4\beta 2$ subtype is the predominant heteromeric nAChR in most of the rat forebrain [105, 106], and it appears to be required for nicotine self-administration in mice [143–145]. The $\alpha 3\beta 4$ receptor appears to be the predominant nAChR in several autonomic ganglia, the trigeminal ganglia [107], the pineal gland [128], and certain areas of the brain [121, 126, 146]. Heterologous expression systems have provided an important tool with which to explore these two simple heteromeric nAChRs in some depth, and the differences in the pharmacological characteristics and functional properties between them are becoming well delineated.

TABLE 4.5 Comparison of EC₅₀ Values of Nicotinic Agonists to Activate Whole Cell Currents Through $\alpha 3\beta 4$ Receptors in Rat Pineal Cells [128] and $^{86}\text{Rb}^+$ Efflux Through $\alpha 3\beta 4$ Receptors Heterologously Expressed in HEK293 Cells [140]

Agonist	EC ₅₀ (μM)	
	$\alpha 3\beta 4$ nAChR in Rat Pineal Cells (Whole Cell Patch Clamp)	$\alpha 3\beta 4$ nAChR in HEK293 Cells ($^{86}\text{Rb}^+$ Efflux)
Epibatidine	0.03	0.06
A-85380	6.0	5.7
DMPP	14	28
Nicotine	21	31
Cytisine	28	24
Acetylcholine	60	110

4.4.2.2 Pharmacology of Mixed Heteromeric nAChRs. The properties of mixed heteromeric nAChRs are less well defined because their more complex subunit structure presents an inherent challenge to their study. For example, the number of receptor subtypes possible in a cell increases exponentially with the number of subunits present. Moreover, no native tissue is known that expresses a single mixed heteromeric subtype exclusively or even predominantly, and cell lines that stably express a single mixed heteromeric receptor exclusively are just now being developed. Nevertheless, some progress has been made in delineating the pharmacology of some mixed heteromeric receptors.

1. *Receptors Containing $\beta 2$ and $\beta 4$ Subunits* Mixed heteromeric nAChRs with the subunit composition of $\alpha 3\beta 2\beta 4$ can be expressed in oocytes and seem to have hybrid properties of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ receptors [111]. Thus, cytisine is a full agonist, as it is in $\alpha 3\beta 4$ receptors, but the receptors are also sensitive to blockade by neuronal bungarotoxin (κ -toxin), another toxin isolated in small quantities from the venom of *B. multicinctus* [147, 148], which appears to be selective for nAChRs containing the $\alpha 3\beta 2$ subunit pair [149].

2. *Receptors Containing $\alpha 5$ Subunits* The $\alpha 5$ subunit in combination with any β subunit does not support channel function or even form an agonist binding site. Therefore, any nAChR that contains $\alpha 5$ subunits would have to also contain another α subunit as well as a β subunit— that is, it would be a mixed heteromeric (see rules of assembly, above). The $\alpha 3\beta 4\alpha 5$ subtype is found in chick ciliary ganglia [50, 62] and in rat superior cervical ganglia, where $\sim 30\%$ of the receptors are $\alpha 3\beta 4\alpha 5$ [108, 150]. Most of the heteromeric nAChRs in chick brain appear to be the $\alpha 4\beta 2$ subtype, but a small fraction ($\sim 15\%$) also contain the $\alpha 5$ subunit [52]. Similarly, most of the nAChRs in rat brain are the $\alpha 4\beta 2$ subtype, but some of these also contain the $\alpha 5$ subunit. Thus, in the rat cerebral cortex $\sim 12\%$ of the $\alpha 4\beta 2$ receptors appear to contain the $\alpha 5$ subunit, whereas in the hippocampus this subunit is found in $\sim 30\%$ of the $\alpha 4\beta 2$ receptors (unpublished).

The presence of the $\alpha 5$ subunit does not appear to markedly affect the nAChR binding site pharmacology [52, 151], but it does alter responses to certain drugs. Thus, deletion of the $\alpha 5$ subunit by antisense oligonucleotide treatment

appears to increase the sensitivity of receptors in chick sympathetic ganglion neurons to acetylcholine, nicotine, and cytisine [152]. On the other hand, inclusion of $\alpha 5$ subunits in $\alpha 3\beta 2$ receptors expressed in oocytes increased their sensitivity to acetylcholine but not to other agonists [153]. Furthermore, the presence of the $\alpha 5$ subunit appears to increase the rate of nAChR desensitization [63, 151, 153]. These effects, combined with the possibility that the $\alpha 5$ subunit affects the trafficking and thus the cellular destination of nAChRs in ganglion neurons [154], suggest that the $\alpha 5$ subunit may significantly influence the physiology as well as the pharmacology of nAChRs. Moreover, recent studies suggest that the presence of the $\alpha 5$ subunit affects the regulation of nAChRs during chronic treatment with nicotine [155].

3. Receptors Containing $\alpha 6$ Subunits Studying potentially important mixed heteromeric nAChRs set among several other, often more numerous, nAChRs is challenging. The $\alpha 6$ -containing receptors are an example of such receptors. The $\alpha 6$ subunit mRNA is expressed in several important and interesting regions in rat brain, including in nuclei of dopamine and norepinephrine neurons [156], which have long been associated with nicotine-stimulated behaviors related to reward responses and locomotion. High levels of this mRNA are also expressed in chick retina [157].

Studies of the $\alpha 6$ receptors have been aided enormously by the use of α -conotoxin MII, one of many small conotoxin peptides discovered in the venoms of predatory marine snails (genus *Conus*). The α -conotoxin group of peptides targets nAChRs with remarkable specificity (for a review, see [158]), and one of these, α -conotoxin MII, has high affinity for $\alpha 6\beta 2^*$ and $\alpha 3\beta 2^*$ nAChRs [159]. Although, it was initially thought to be selective for $\alpha 3\beta 2^*$ receptors, subsequent studies using knockout mice lacking the $\alpha 6$ subunit gene indicate that the $\alpha 6\beta 2^*$ subtype is its most likely target in most brain areas [160]. Early studies with α -conotoxin MII showed that it blocked 30–50% of nicotine-stimulated dopamine release in rat striatal tissues in vitro [161, 162], indicating an important role for $\alpha 6\beta 2^*$ nAChRs in this well-established effect of nicotine. Subsequently, the $\beta 3$ subunit was shown to be an integral component of these α -conotoxin MII-sensitive receptors [163, 164].

Synthesis of an [125 I] α -conotoxin MII analog allowed the distribution of $\alpha 6$ -nAChRs in brain to be mapped by autoradiography [127]. These mapping studies showed that the $\alpha 6$ receptors have a relatively narrow distribution in the brain. Their highest densities are in the retina and brain areas to which the visual tract projects, such as the superior colliculus and lateral geniculate nuclei [54, 56, 102, 127, 160]. Relatively high densities of $\alpha 6$ -nAChRs are also found in the striatum and nucleus accumbens [127, 160, 165]. In fact, $\alpha 6\beta 2^*$ nAChRs are found on the dopamine axon terminals of rats, mice, and primates [55, 161, 165–169], accounting for their involvement in nicotine-stimulated dopamine release measured in vitro. The $\alpha 6$ subunit appears capable of forming one or more mixed heteromeric receptors with $\alpha 4$, $\beta 2$ and $\beta 3$ subunits [55]; therefore, further studies are required to determine the complete subunit compositions of the nAChR subtypes involved in dopamine release, but one leading candidate would be the $\alpha 6\beta 2\beta^*$ subtype.

Other α -conotoxins appear to be specific for other nAChR subtypes. For example, α -conotoxin AuIB appears to be specific for $\alpha 3\beta 4^*$ nAChRs [170], and studies with it have helped to establish roles for these receptors in the rat medial habenula [146]. In

addition, nicotine-stimulated norepinephrine release in rat hippocampus synaptosomes has a different pharmacological profile compared to dopamine release in the striatum [171]. The agonist potencies and particularly the efficacy of cytisine suggested that the norepinephrine release was mediated by $\alpha 3\beta 4^*$ receptors [171]. This suggestion was supported by later studies with α -conotoxin AuIB in vitro [170], as well as by studies that showed that in vivo microinjection of α -conotoxin AuIB into the locus ceruleus, the cell body origin of the norepinephrine axons, partially blocked the release of norepinephrine [172]. Interestingly, these studies found that microinjected α -conotoxin MII also partially blocked norepinephrine release, suggesting that $\alpha 6\beta 2$ (or $\alpha 3\beta 2$) nAChRs are also involved, or possibly a mixed heteromeric subtype comprised of an α subunit in association with both $\beta 2$ and $\beta 4$ subunits [172].

Determining the subunit compositions of nAChRs involved in important functions is a critical step in delineating the pharmacology of those receptors. The studies of the $\alpha 6$ nAChRs involved in dopamine release illustrate how a multipronged approach utilizing selective ligands, subunit-selective antibodies, and knockout mice can be brought to bear on the question of which nAChR subtypes is involved in a specific function. Because nAChRs appear to mediate the release of several different neurotransmitters in brain pathways, they are implicated in diverse and important functions, such as the reward pathways associated with dopamine, arousal and motivation associated with norepinephrine, anxiolytic responses associated with GABA, and cognitive processes associated with acetylcholine and glutamate. In some cases there appears to be considerable redundancy built into these functions. For example, nicotine-stimulated dopamine release from striatal synaptosomes is mediated by at least two different nAChR subtypes (the leading candidates are $\alpha 6\beta 2\beta 3^*$ and $\alpha 4\beta 2^*$), both of which are located on axons of dopaminergic neurons [55, 164, 167]. But interestingly, dopamine release stimulated by systemic injections of nicotine in rats and mice in vivo appears to originate at somatodendritic nAChRs located on the midbrain dopamine neuron cell bodies [167, 173, 174]. Moreover, receptors containing the $\alpha 6$ subunit do not seem to be required for this effect of systemic nicotine [167]. The full range of the physiological roles of these different nAChRs in the dopamine pathway is not known, but this is a crucial question that bears on the potential of these receptors as targets for drug interventions in disorders as diverse as Parkinson's disease, schizophrenia, Tourette's disease, attention-deficit disorders, and, of course, nicotine addiction.

4.4.2.3 Distribution of Heteromeric nAChRs. In addition to delineating the pharmacological profiles of the nAChR subtypes, binding studies have uncovered useful tools for probing nAChRs in the CNS. For example, the total population of heteromeric nAChRs can be mapped in autoradiographic studies with [125 I]EB, which labels all subtypes with very high affinity (50–300 pM). Alternatively, because of its high affinity and selectivity, [125 I]5-iodo-A-85380 can be used to label only nAChRs that contain $\beta 2$ subunits [121, 175]. Figure 4.2 compares the autoradiographic images of [125 I]EB and [125 I]A-85380 binding to nAChRs in adjacent sagittal sections of rat brain. Note the similarity in the distribution of the binding sites, but also note that [125 I]EB prominently labels the pineal gland and the fasciculus retroflexus (Fig. 4.2a), whereas [125 I]A-85380 does not (Fig. 4.2b). The presence of the $\beta 4$ -containing receptors is emphasized in Figure 4.1c, in which the tissue sections

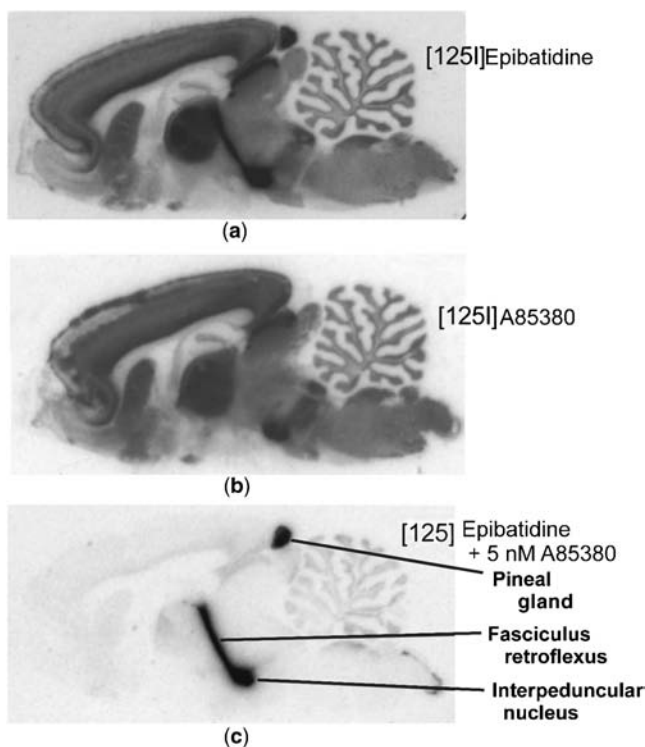


Figure 4.2 Autoradiographic images of nAChRs labeled by $[^{125}\text{I}]\text{epibatidine}$ and $[^{125}\text{I}]\text{A-85380}$. nAChRs labeled by $[^{125}\text{I}]\text{epibatidine}$ and $[^{125}\text{I}]\text{A-85380}$ in adjacent sagittal sections of rat brain are shown in (a) and (b) respectively. In (c), the differences between the binding sites labeled by these two radioligands are emphasized by labeling adjacent sections with $[^{125}\text{I}]\text{epibatidine}$ in the presence of unlabeled A-85380 to mask all binding sites containing $\beta 2$ subunits. Adapted from [121].

were incubated with $[^{125}\text{I}]\text{EB}$ in the presence of 5 nM unlabeled A-85380 to mask the nAChR binding sites that contain $\beta 2$ subunits.

4.5 REGULATION OF nAChRs

4.5.1 Desensitization and Inactivation of nAChRs

During exposure to agonists, neuronal nAChRs desensitize rapidly, becoming unresponsive for a period of time. This phenomenon, which closely resembles the desensitization of muscle nAChRs described by Katz and Thesleff [176], is seen in virtually all neuronal nAChRs that have been examined. In the desensitized state nAChRs have much higher affinity for agonists than they do in their resting (activatable) state, which accounts for why the affinities of agonists measured in binding studies are so much higher than their potencies measured in studies of receptor function would predict. Moreover, in the prolonged presence of agonists, more receptors are recruited into the desensitized state. Consistent with this, agonists

are more potent in desensitizing nAChRs than in activating them both in vitro [137, 140, 142, 177–181] and in vivo [182, 183].

The difference in the potencies of nicotine to desensitize versus activate nAChRs depends on the receptor subtype. For example, nicotine was ~ 50 times more potent in desensitizing than in activating $\alpha 3\beta 4$ receptors expressed in frog oocytes, whereas it was > 200 times more potent in desensitizing than activating the $\alpha 4\beta 2$ subtype [181]. In mammalian cells expressing nAChRs, nicotine was ~ 9 times more potent in desensitizing $\alpha 3\beta 4$ receptors than in activating them [140], and again this difference in potency was much greater at $\alpha 4\beta 2$ receptors [137, 142]. In vivo, nicotine is ~ 5 times more potent in desensitizing nicotine-induced prolactin release in the rat than in stimulating the response [183].

The rate of onset of desensitization by agonists is concentration dependent and also varies among receptor subtypes [184–186]. The rate of recovery from desensitization depends on the concentration of agonist and in some cases on the duration of exposure [140, 187, 188]. Just as important, the rate of recovery from desensitization also varies with subtype. For example, the $\alpha 3\beta 4$ subtype appears to recover faster than the $\alpha 4\beta 2$ subtype from nicotine-induced desensitization [181].

Desensitization of nAChRs by acetylcholine released by neurons in vivo, if it occurs at all, is brief because the agonist is rapidly hydrolyzed by cholinesterase. But following administration of nicotine or other agonists that are not rapidly degraded, desensitization lasts much longer, as the agonist continues to circulate, bind to receptors, and presumably recruit more of them into the desensitized state. For example, a single injection of nicotine, which has a plasma half-life of < 60 min in the rat [189], can produce profound desensitization of some CNS responses that lasts for several hours or longer [183, 190]. In fact, because of this prolonged desensitization, nicotine's predominant effect over time (i.e., its time-averaged effect), at least at some receptors, is that of an antagonist [142, 183, 187].

The degree to which a drug behaves as a time-averaged antagonist after a single acute administration depends on its affinity for a particular receptor subtype as well as its rate of removal from nearby cells, where it could be sequestered and then slowly released to maintain receptor desensitization [191]. Because nicotine and most other agonists have much higher affinity for $\alpha 4\beta 2^*$ than for $\alpha 3\beta 4^*$ nAChR subtypes, functions mediated by $\alpha 4\beta 2^*$ receptors, which are the predominant subtype in the CNS, are probably more affected by desensitization than are the $\alpha 3\beta 4^*$ receptors in the ganglia; moreover, nicotine concentrations in the brain typically exceed those in plasma [192, 193]. The result is that under conditions of repeated administration of nicotine at the doses taken in by a cigarette smoker, most of the $\alpha 4\beta 2^*$ receptors in the brain are probably desensitized nearly all of the time; the probable exception is after overnight abstinence from smoking (i.e., ~ 4 – 6 half-lives for plasma nicotine), when a significant fraction of receptors would be expected to recover function.

In addition to classical receptor desensitization, which reverses over a time frame of seconds to minutes after removal of the agonist, a more extended loss of receptor function may take place during prolonged or repeated exposure to nicotine or other agonists. This more durable desensitization, which is referred to as receptor *inactivation*, has been seen to varying degrees in nAChRs in electric tissue [194], rat PC12 cells [177, 195, 196], human SH-SY5Y cells [187], rodent brain tissues [178, 197], and rats in vivo [182]. Inactivation is also found in heterologously expressed receptors [140, 198–200], suggesting that it is an intrinsic property of the receptor,

like acute desensitization. In fact, it is even possible that rather than being a distinct condition or engaging different mechanisms, inactivation may simply be protracted desensitization resulting from slow escape of a sequestered agonist like nicotine from cells near the receptor [191]. In either case, $\alpha 3\beta 4$ receptors again recover function rather rapidly even from prolonged exposure to agonists. For example, after a five-day exposure to a high concentration of nicotine, $\alpha 3\beta 4$ receptors heterologously expressed in mammalian cells recover function with a half-time of 11 min [140]. The rapid recovery of $\alpha 3\beta 4$ receptor activity may explain why ganglionic functions can be maintained fairly well between the episodes of self-administration of nicotine that occurs over long periods of time in a smoker.

During the periods that $\alpha 4\beta 2^*$ receptors are desensitized, the $\alpha 3\beta 4^*$ receptors in brain, as well as any other receptor subtypes that desensitize less readily or recover more quickly, probably take on added functional significance. For example, during those periods that nicotine cannot stimulate dopamine release via the desensitized $\alpha 4\beta 2^*$ receptors, it might still be capable of stimulating norepinephrine release, which appears to be mediated by $\alpha 3\beta 4^*$ receptors [170, 171]. These factors could be particularly important to the mechanisms related to induction and/or maintenance of nicotine addiction as well as to the physiological and subjective effects elicited by withdrawal from nicotine.

4.5.2 Agonist-Induced Up-regulation of nAChRs

The density of nAChRs increases during chronic exposure to nicotine or other agonists. This unusual characteristic, referred to as receptor up-regulation, was first seen as an increase in nAChR binding sites in brains from rats and mice treated in vivo with nicotine for 10–14 days [201–203], but nicotine-induced up-regulation has since been found in cultured cells expressing virtually every nAChR subtype and subunit combination examined [123, 138, 140, 187, 204–206]. Moreover, as shown in Figure 4.3, nAChR binding sites are also increased in autopsied brains from people who smoked compared to the binding sites in brains from age-matched controls [207–209]. The clear implication is that the nicotine-induced increase in brain nAChRs may be linked to nicotine addiction.

Nicotine exposure increases nearly all nAChR subtypes expressed by cells in culture; in contrast, the nicotine-induced increase in nAChRs in vivo is much more limited. For example, chronic nicotine treatments that increase nAChRs in rat brain do not increase the receptors in any peripheral tissues examined, including superior cervical ganglia, adrenal gland, or pineal gland [210, 211]. The reasons for this difference in regulation between CNS and peripheral nAChRs during chronic exposure to nicotine are not yet completely known, but the receptors increased in brain by nicotine treatments are the $\alpha 4\beta 2^*$ subtype [106], whereas the nAChRs in the peripheral tissues are predominantly $\alpha 3\beta 4^*$ receptor subtypes. Thus, the higher affinity of nicotine for the $\alpha 4\beta 2^*$ receptors as well as the higher nicotine concentration reached in brain during chronic administration could both contribute to the difference in regulation. Furthermore, an extracellular domain of the $\beta 2$ subunit itself may be critical to the mechanisms that underlie the up-regulation [212].

Perhaps as intriguing as the differences in regulation between nAChR subtypes in brain and periphery is that the increase in $\alpha 4\beta 2^*$ receptors appears to vary across brain regions and even between neighboring brain nuclei [213–215]. This suggests

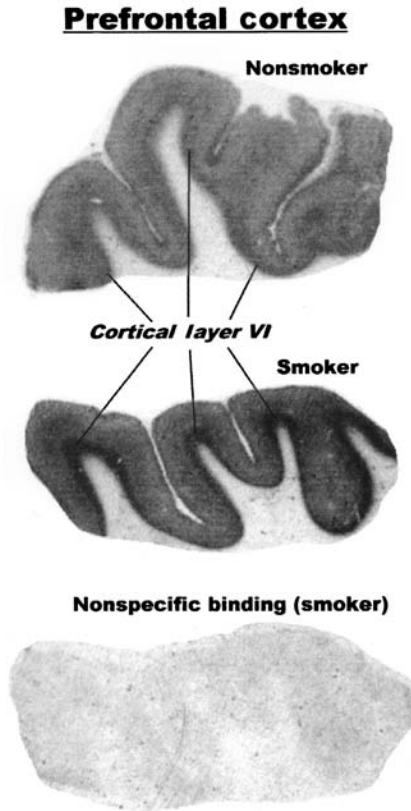


Figure 4.3 Autoradiographic images of nAChRs labeled by [^3H]epibatidine in human frontal cerebral cortex from a nonsmoker and a smoker. These images are representative of the increases in nAChRs that are found in brains from smokers. The lower figure depicts the very low level of nonspecific binding with [^3H]epibatidine. From [209].

that there are differences among the $\alpha 4\beta 2^*$ receptors that affect their susceptibility to up-regulation by nicotine. One possibility is that the presence of an additional subunit in a mixed heteromeric $\alpha 4\beta 2^*$ receptor affects the up-regulation. For example, $\sim 15\%$ of the $\alpha 4\beta 2^*$ receptors in rat cerebral cortex and $\sim 25\%$ of the receptors in hippocampus contain an $\alpha 5$ subunit, and the receptors that contain these subunits do not appear to be up-regulated by chronic nicotine [155]. Another possibility is that factors intrinsic to particular neurons (e.g., the rate of receptor turnover) affect the degree to which nicotine can up-regulate the receptors.

Interestingly, a recent study found that $\alpha 6$ -containing nAChRs, which had not been examined in earlier studies, were increased in several areas of brain, including the striatum, from rats that had been trained to chronically self-administer nicotine intravenously [216]. In contrast, in mice that had received nicotine chronically via continuous infusion or in their drinking water [168, 217] and in rats that received nicotine via osmotic mini-pumps [218], the $\alpha 6$ -containing receptors in the striatum were actually decreased. It is probable that technical differences in the receptor measurements or possibly differences between species or rat strains account for these

opposite results for the $\alpha 6$ receptors (but it would be particularly interesting if the method of nicotine administration—voluntary self-administration versus nonvoluntary infusions—influences its effects on receptors).

The nicotine-induced increase in nAChR binding sites in cells in culture is usually associated with increased receptor function [134, 138, 142, 206, 219]; however, there are also instances where the receptor binding sites are increased but function appears to be nearly absent [220]. In brain tissues from rats treated chronically with nicotine, some studies have found increased nAChR-mediated responses corresponding to the increased receptor binding sites [221–224], but other studies using brain tissues from rat or mouse found either no increase or even a decrease in nAChR-mediated responses [225–227]. To complicate this picture further, differential effects on nicotine-stimulated release of two or more neurotransmitters were found in brain tissues from the same chronically treated rats [222, 224]. Moreover, in a study designed to measure a CNS nAChR-mediated response in vivo, nicotine-stimulated prolactin release in rats was abolished for more than a week after chronic administration of nicotine [182].

Some of the impressive variability in the results of studies that have assessed function after chronic administration of nicotine in vivo might be explained by the fact that different nAChR subtypes are associated with the release of different neurotransmitters. For example, nicotine-stimulated norepinephrine release in the hippocampus is mediated at least in part by an $\alpha 3\beta 4^*$ subtype [170, 171], while nicotine-stimulated dopamine release from striatal synaptosomes appears to be mediated by an $\alpha 4\beta 2^*$ subtype, which is presumably increased by chronic administration of nicotine, as well as an $\alpha 6\beta 2^*$ subtype that appears to be decreased by chronic nicotine [168, 217, 218]. Moreover, the variability in the results of the functional status of nAChRs after chronic administration of nicotine probably also reflects an interplay between an increase in $\alpha 4\beta 2^*$ receptors and reversible desensitization that prevents their function. If that is the case, after chronic administration, there might be only a window in time when an increase in receptor function would be measurable. This window would correspond to the period when the receptors have recovered from desensitization but are still increased. Furthermore, it is possible that there are factors at work here, either technical (e.g., incomplete washout of nicotine from brain tissues in vitro) or pharmacokinetic (e.g., long-term sequestration of nicotine in brain), that in some cases might not have been fully accounted for in assessing functional responses after chronic administration of nicotine.

Despite their potential for function, the increased $\alpha 4\beta 2$ receptors in a smoker are probably desensitized most of the time. This leads to the interesting possibility that self-administration of nicotine may be impelled by the need to prevent the withdrawal state by keeping the receptors desensitized, thus preventing the increased activity that would occur if the up-regulated receptors were fully functional. This could set up an oscillation between overstimulation of critical brain circuits due to the increased receptors followed rapidly by underactivity of the same circuits due to receptor desensitization. If that happens, it is not hard to imagine how it could maintain the repetitive, self-reinforcing behavior we call addiction.

Although it is not known how an increase in brain receptors affects complex behaviors, nicotine-induced up-regulation of nAChRs is an unusual and intriguing phenomenon, and the cellular/molecular mechanisms underlying the increase in receptors are the focus of much research. The increase in brain $\alpha 4\beta 2$ nAChRs induced by nicotine does not depend on increased transcription of mRNA [204,

214, 228], which obviously places posttranscriptional events at the center of focus. The mechanisms that have been proposed to explain nicotine-induced upregulation of $\alpha 4\beta 2$ nAChRs are derived from studies of receptors heterologously expressed in mammalian cells. These mechanisms include (1) slower removal and degradation of cell surface receptors [134, 204, 229], (2) increased assembly of receptors [134, 142, 212, 228, 230, 231], (3) increased insertion of receptors onto the cell surface [232], and (4) altered conformation of cell surface receptors that results in their stabilization in a state with high affinity for and sensitivity to agonists [229, 233].

Each of these mechanisms could theoretically explain the observations of increased binding and function of receptors during exposure to nicotine, but the most compelling data so far, derived from $\alpha 4\beta 2$ receptors heterologously expressed in mammalian cells, point to a combination of effects of nicotine. The first effect is increased maturation and final assembly of receptor pentamers in the endoplasmic reticulum. According to this hypothesis, nicotine (and other agonists) can bind to an immature $\alpha 4\beta 2$ subunit oligomer, perhaps a tetramer, and act as a chaperone to promote the introduction of the third $\beta 2$ subunit into the receptor, following which the pentamer undergoes final processing and transport to the cell surface as a mature, functional receptor¹ [142, 231]. The promotion of receptor assembly by nicotine is at least in part dependent on a “microdomain” in the $\beta 2$ subunit near the interface with the α subunit [212], which might account for the observation that in vivo $\alpha 4\beta 2$ but not $\alpha 3\beta 4$ receptors are increased.

In addition to promoting increased assembly of $\alpha 4\beta 2$ nAChRs, nicotinic agonists appear to slow their removal from the cell surface and their degradation [142, 204]. The mechanism that results in stabilization of $\alpha 4\beta 2$ receptors is probably no less important than increased assembly, but it is not yet known. It is possible, however, that the fully assembled receptors on the cell surface are less tempting or accessible targets for degradative enzymes. On the other hand, if nicotine affects receptor assembly and stability independently, it could explain why $\beta 4$ -containing receptors expressed in cultured cells are increased by nicotine, but not to the same extent as $\beta 2$ -containing receptors [123].

4.6 CONCLUSIONS

As nAChRs enter the second century since their discovery, we know a great deal about their subunit structure, channel function, and regulation. Their crucial physiological role in the autonomic nervous system is well established and their many potential roles in the CNS are becoming clearer. In the limited number of autonomic and sensory ganglia that have been examined, $\alpha 3\beta 4^*$ receptors predominate. In the CNS, although there are potentially a large number of receptor subtypes based on possible subunit combinations, a relatively small number of subtypes seems to be highly represented

¹Since the site of action of nicotine to increase assembly of receptors is intracellular, this mechanism indicates that even charged quaternary amine nicotinic agonists, like carbamylcholine, which would not be expected to readily cross the cell membrane to reach the endoplasmic reticulum to promote increased assembly, probably do get internalized in cells, albeit slowly, over the course of the several hours to days of incubation [142, 231].

in most regions. These include the homomeric $\alpha 7$ receptor and several heteromeric receptors comprising the $\alpha 4\beta 2^*$ and to a lesser extent $\alpha 3\beta 4^*$ subtypes. Although all nAChRs, but especially the heteromeric subtypes, share many pharmacological characteristics, some ligands and drugs have emerged that can distinguish among the subtypes, especially between those containing $\beta 2$ versus $\beta 4$ subunits. The likely crucial involvement of nAChRs in nicotine addiction makes them an important target for intervention in this disorder. Furthermore, because nAChRs in the CNS seem to be strategically located to modulate the release of several important neurotransmitters, including dopamine, norepinephrine, acetylcholine, GABA, and glutamate, they may influence a wide range of CNS functions and pathways. Consequently, drugs aimed at nAChR subtypes have great therapeutic potential in conditions as diverse as cognitive disorders, attention-deficit disorders, schizophrenia, Tourette's syndrome, Parkinson's disease, and neuropathic pain. It is therefore very likely that the pharmacology of these receptors will continue to be of great interest well into the next 100 years.

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5

MUSCARINIC ACETYLCHOLINE RECEPTORS

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5.1	Introduction	148
5.2	Distribution of mAChRs	149
5.3	General Structural Features of mAChRs	149
5.4	Muscarinic Ligands and Mechanisms Involved in Ligand Binding	152
5.4.1	Agonists	152
5.4.2	Antagonists	152
5.4.3	Allosteric Ligands	153
5.4.4	Muscarinic Drugs in Current Clinical Use	154
5.4.5	Muscarinic Drugs: Potential Future Clinical Applications	154
5.5	Mechanisms Involved in mAChR Activation	155
5.6	G-Protein coupling Properties of mAChRs	155
5.7	Structural Basis of Selectivity of mAChR–G Protein Interactions	157
5.8	Modulation of mAChR–G Protein Coupling by RGS Proteins	158
5.9	Signaling Pathways Modulated by mAChR Activation	158
5.10	Modulation of MAPK Pathways	159
5.11	Ion Channels	160
5.11.1	Closure of Potassium Channels	160
5.11.2	Opening of Potassium Channels	160
5.11.3	Modulation of Calcium Channels	161
5.11.4	Nonselective Cation Channels	161
5.11.5	Chloride Channels	161
5.12	Regulation of mAChR Activity	162
5.12.1	Receptor Uncoupling	162
5.12.2	Receptor Internalization	162
5.12.3	Receptor Downregulation	163
5.12.4	Receptor Phosphorylation	164
5.12.4.1	Protein Kinase C	164
5.12.4.2	GPCR Kinases	164
5.12.5	Mechanisms Involved in mAChR Resensitization	166
5.13	mAChR Dimerization	166
5.14	Phenotypical Analysis of mAChR-deficient Mice	167

5.14.1	Functions Mediated by Central mAChRs	168
5.14.1.1	Learning and Memory	168
5.14.1.2	Epileptic Seizures	169
5.14.1.3	Locomotor Activity	169
5.14.1.4	Analgesia	170
5.14.1.5	Muscarinic Agonist-induced Tremor and Hypothermia	170
5.14.1.6	Role of M ₃ mAChRs in Stimulating Food Intake	171
5.14.1.7	Central Muscarinic Autoreceptors	171
5.14.1.8	Modulation of Striatal Dopamine Release	171
5.14.1.9	Suppression of Inhibitory Hippocampal Synapses	172
5.14.1.10	M ₄ Receptor-mediated Regulation of Prepulse Inhibition and Haloperidol-Induced Catalepsy	172
5.14.1.11	Role of M ₄ and M ₅ Receptors in Modulating Dopamine Efflux in Nucleus Accumbens	172
5.14.1.12	Role of M ₅ Receptors in Modulating Rewarding Effects of Drugs of Abuse	173
5.14.2	Functions Mediated by Peripheral mAChRs	173
5.14.2.1	Smooth Muscle	173
5.14.2.2	Salivary Secretion	174
5.14.2.3	Insulin and Glucagon Secretion from Pancreatic Islets	174
5.14.2.4	Amylase Secretion from Exocrine Pancreas	175
5.14.2.5	Gastric Acid Secretion	175
5.14.2.6	Cardiovascular System	175
5.14.2.6.1	Heart	175
5.14.2.6.2	Blood Vessels	176
5.14.2.6.3	Cardiovascular Effects of McN-A-343	176
5.14.2.7	Peripheral Muscarinic Autoreceptors and Heteroreceptors	176
5.14.2.8	Role of M ₁ mAChRs in Development of Cytolytic T Cells	177
5.14.2.9	Functions of mAChRs in Skin	177
5.15	Conclusions	177
	Acknowledgments	177
	References	178

5.1 INTRODUCTION

Acetylcholine (ACh) is one of the major neurotransmitters in both the central and peripheral nervous systems. Historically, the effects of ACh that are mimicked by the alkaloid muscarine and that are selectively blocked by atropine are referred to as muscarinic effects. Molecular cloning studies have shown that the muscarinic effects of ACh are mediated by a family of cell surface receptors referred to as muscarinic ACh receptors (M₁–M₅ mAChRs; [1–3]).

mAChRs modulate the activity of many fundamental central and peripheral functions. In the central nervous system (CNS), mAChRs play important roles in most cognitive functions and many key motor, autonomous, sensory, and behavioral processes [4–6]. Importantly, disturbances in the central muscarinic cholinergic system have been implicated in several pathophysiological conditions, including Alzheimer's and Parkinson's disease, depression, epilepsy, and schizophrenia [7, 8].

In the body periphery, mAChRs mediate the well-known functions of ACh at parasympathetically innervated effector organs, including slowing of the heart, contraction of smooth muscle, and stimulation of glandular secretion [4, 9].

5.2 DISTRIBUTION OF mAChRs

The individual mAChRs are widely expressed in nearly all parts of the CNS and the body periphery [10–12]. Characteristically, each tissue or organ expresses multiple mAChR subtypes, with only very few exceptions (the heart almost exclusively expresses M_2 receptors). Detailed immunocytochemical studies in rat hippocampus [13] and striatum [14] have shown, for example, that multiple mAChR subtypes are expressed on specific neuronal populations both pre- and postsynaptically. It is likely that similarly complex expression patterns are present in most other regions of the brain and in many peripheral tissues.

The distribution patterns of the M_1 – M_5 mAChRs have been described in several excellent reviews ([10–12, 15]; see also Table 5.1). In brief, M_1 mAChRs are present at particularly high levels in the forebrain, including cerebral cortex, hippocampus, and striatum. M_2 receptors are widely expressed both in the CNS and in the body periphery, particularly in the heart and in smooth muscle tissues. Like the M_2 receptor, the M_3 mAChR subtype is widely expressed in different regions of the brain and in peripheral organs, including smooth muscle and glandular tissues. M_4 receptors are preferentially found in the CNS, particularly in different areas of the forebrain. M_5 receptors are expressed, at rather low levels, in both neuronal and nonneuronal cells [15]. Interestingly, M_5 receptor messenger ribonucleic acid (mRNA) represents the only mAChR mRNA that can be detected in dopaminergic neurons of the midbrain [19, 20].

5.3 GENERAL STRUCTURAL FEATURES OF mAChRs

The M_1 – M_5 mAChRs are prototypical members of the superfamily of class I G-protein-coupled receptors (GPCRs; [1–3]). Class I GPCRs, which are also referred to as rhodopsinlike receptors, share about 20 highly conserved amino acids (underlined in Fig. 5.1). The structural hallmark of mAChRs and all other GPCRs is the presence of seven α -helically arranged transmembrane domains (TMs I–VII; Fig. 5.1) which form a tightly packed transmembrane core. The N-terminal portion of the receptor protein is located extracellularly, whereas the C-terminal segment protrudes into the cytoplasm. The seven transmembrane helices are linked by three intracellular (i1–i3; Fig. 5.1) and three extracellular loops (o2–o4; Fig. 5.1).

At present, bovine rhodopsin, in its inactive state, is the only GPCR for which high-resolution structural information is available [24]. Molecular modeling studies suggest that the M_1 – M_5 mAChRs share a high degree of structural similarity with that of the rhodopsin template, especially within the regions endowed with secondary structure [25, 26]. A characteristic structural feature of the mAChRs is the presence of a rather large i3 loop (157–240 amino acids in length), which, except for the N- and C-terminal segments, displays virtually no sequence homology among the different subtypes. The N- and C-terminal portions of the i3 loop play important roles in

TABLE 5.1 mAChR Family: Pharmacological and Functional Properties and Anatomical Distribution by mAChR Subtype

Property	M ₁	M ₂	M ₃	M ₄	M ₅
Number of aminoacids (human)	460	466	590	479	532
Chromosomal localization ^a	11q12-13	7q35-36	1q43-44	11p12-11.2	15q26
GenBank/EMBL accession number	X15263	X15264, M16404	X15266	X15265, M16405	M80333
Subtype-preferring antagonists (examples) ^b	Pirenzepine MT7 ^c	Tripitramine	Darifenacin	PD102807 MT3 ^c	— ^d
G-protein coupling selectivity	G _{q/11}	G _{i/o}	G _{q/11}	G _{i/o}	G _{q/11}
Distribution ^c					
CNS					
Cerebral cortex	+	+	+	+	
Hippocampus	+	+	+	+	(+)
Striatum	+	+	+	+	(+)
Thalamus	+	+	+	+	
Brain stem and cerebellum	(+)	+	+	(+)	
Spinal cord		+		(+)	
Midbrain dopaminergic neurons					+
Vascular endothelium of cerebral arteries and arterioles					+
<i>Peripheral tissues</i>					
Heart		+			
Smooth muscle		+	+	(+)	
Exocrine glands	+		+		
Pancreatic islets	(+)		+		
Vascular endothelium			+		

^aFrom [15a].

^bExcept for certain snake toxins, such as MT3 or MT7, the degree of subtype preference of so-called subtype-selective muscarinic antagonists is usually relatively small (for a summary of affinity values, see [3]).

^cMT3 and MT7 are toxins isolated from the venom of the green mamba (*Dendroaspis augusticeps*) [16, 17].

^dM₅ mAChRs are pharmacologically distinct in that they display very low affinity for antagonists such as AF-DX 116 and its derivatives [3, 18].

^eNote that mAChRs are present in nearly all cell types, tissues, and/or organs. Summarized here are the mAChR expression patterns for some key tissues or organs. The symbols + +, +, and (+) indicate relative mAChR protein and/or mRNA expression levels. However, relative mAChR expression levels may differ among species.

receptor – G protein coupling [2, 22], whereas the central portions of the i3 loop are involved in the regulation of receptor activity (see below). The five receptor subtypes share the highest degree of sequence homology with the seven membrane-spanning domains which are known to be involved in ACh binding [2, 25]. The three-dimensional organization of the mAChR proteins and the potential functional roles of several conserved structural features such as a disulfide bond connecting the top of TM III with the o3 domain, the attachment of sugar chains at N-terminal Asn residues, and the palmitoylation of Cys residues present in the C-terminal tail have been reviewed in detail previously [2].

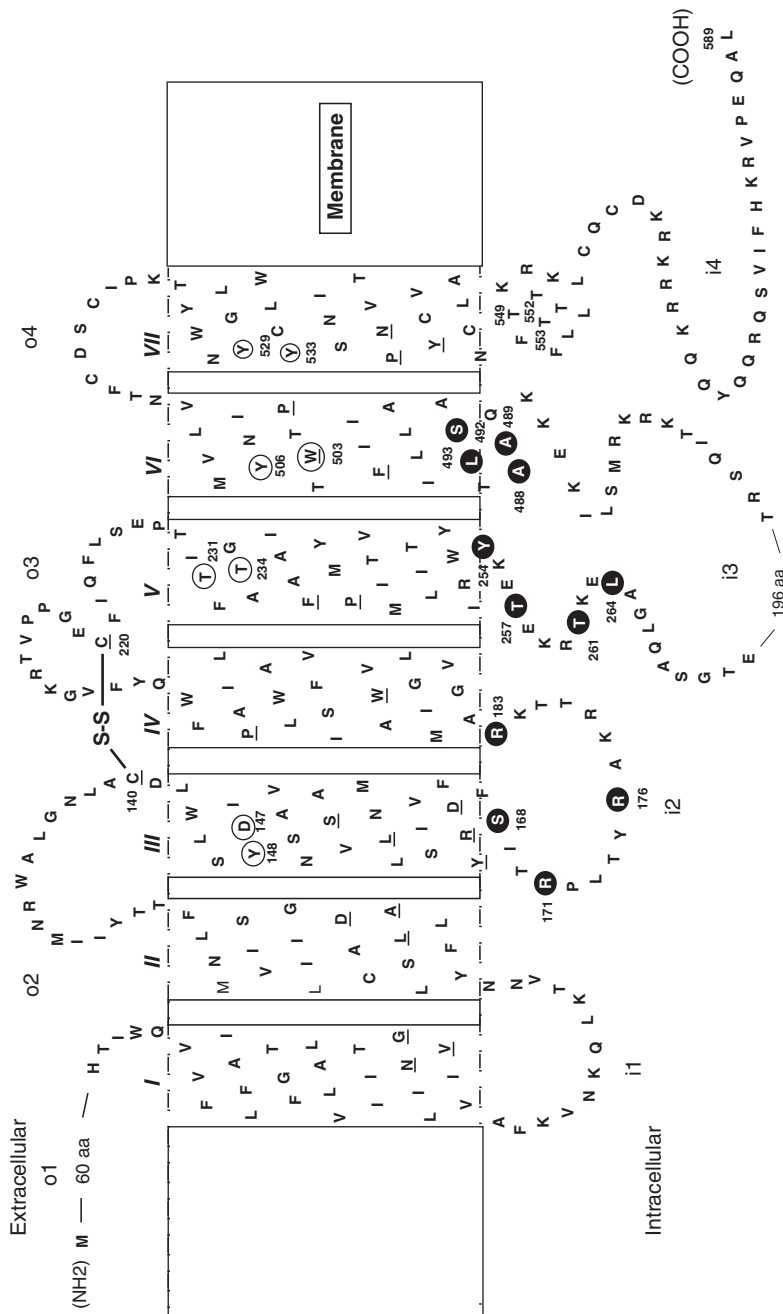


Figure 5.1 Predicted transmembrane topology of rat M_3 mAChR showing functionally important residues. Like all other GPCRs, the M_3 mAChR consists of an extracellular N-terminal domain (o1), seven transmembrane domains (TMs I–VII) connected by three extracellular (o2–o4) and three intracellular (i3) loops, and an intracellular C-terminal tail (i4). Amino acids which are highly conserved among class I GPCRs are underlined. Many of these amino acids play key roles in the agonist-induced conformational activation of the receptor protein [2, 21, 22]. Residues highlighted by open circles are predicted to be involved in the binding of ACh and other classical muscarinic agonists [2]. The amino acids highlighted by filled circles have been shown to determine the ability of the M_3 mAChR to selectively interact with G proteins of the $G_{q/11}$ family [2, 21, 22]. Only the membrane-proximal portions of the o1 and i3 domains are shown. Numbers indicate amino acid positions within the rat M_3 mAChR sequence [23].

5.4 MUSCARINIC LIGANDS AND MECHANISMS INVOLVED IN LIGAND BINDING

5.4.1 Agonists

Site-directed mutagenesis, biochemical, and molecular modeling studies indicate that ACh binds to the M₁–M₅ receptors within a cleft enclosed by the ringlike arrangement of TMs I–VII about 10–15 Å away from the membrane surface [2, 25]. The amino acids involved in ACh binding are located on different TM helices, primarily on TMs III, V, VI, and VII ([2, 25]; Fig. 5.1). Importantly, the positively charged ammonium head group of ACh (or the amino/ammonium head group of other classical muscarinic agonists or antagonists) is engaged in an ion–ion interaction with a TM III aspartate residue (D147 in Fig. 5.1) which is conserved among all biogenic amine GPCRs. This ion pair is surrounded by a cluster of aromatic amino acids located on TMs III, VI, and VII, thus creating a charge-stabilized aromatic cage [2, 25]. The specificity of ACh–mAChR binding depends on specific interactions of the ACh side chain with mAChR-specific amino acids facing the central ligand binding pocket. The molecular identity of these amino acids and their proposed interactions with the ACh molecule or related muscarinic agonists have been the subject of several recent reviews ([2, 25, 27]; also see Fig. 5.1).

Since the amino acids lining the ACh binding cavity are highly conserved among the M₁–M₅ mAChRs, the development of agonists or antagonists endowed with a high degree of mAChR subtype selectivity has proven to be a very difficult task. At present, agonists targeted at the classical ACh binding site that preferentially bind to a particular mAChR subtype are not available [3].

Several muscarinic agonists have been identified that display functional selectivity for a specific mAChR subtype, such as the M₁ mAChR, at least under certain experimental conditions. In almost all cases, these compounds are partial agonists, such as McN-A-343 (4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butyryl]trimethylammonium chloride) and its derivatives [28], which usually show similar affinities for the different mAChR subtypes but preferentially activate one or more specific mAChRs, at least under certain experimental conditions, reflecting differences in efficacy. However, since the activity pattern of partial agonists greatly depends on receptor density, cell type, and actual response studied, it is difficult to predict what kind of functional activity/selectivity these agents will display *in vivo*.

5.4.2 Antagonists

The binding pocket of classical muscarinic antagonists, including atropine, scopolamine, or quinuclidinyl benzilate, partially overlaps with that of ACh and classical muscarinic agonists. However, antagonists usually form additional strong interactions with hydrophobic receptor residues, thus stabilizing the inactive state of the receptor [2, 25].

In general, the degree of selectivity of so-called mAChR subtype-selective muscarinic antagonists which are currently used to distinguish between different mAChR subtypes is rather limited [3]. Such compounds include, for example, pirenzepine (M₁ receptor preferring), triptamine and SCH 57790 (4-Cyclohexyl- α -[4[[4-methoxyphenyl]sulphonyl]-phenyl]-1-piperazineacetonitrile; M₂ receptor preferring), darifenacin (M₃ receptor preferring), or PD 102807 (3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-12H-isoquino[1,2-b]pyrrolo[3,2-f][1,3]benzoxazine-1-carboxylic acid ethyl

ester M_4 receptor preferring) (Table 5.1). Antagonists that preferentially bind to M_5 receptors are not available at present.

As a general rule, multiple subtype-preferring muscarinic antagonists need to be applied to define the mAChR(s) mediating a specific physiological function. This approach has been highly useful to define the physiological functions of distinct mAChRs in *in vitro* studies [3]. In contrast, the use of this strategy in *in vivo* studies has frequently led to conflicting results, probably due the fact that antagonist potency *in vivo* does not only depend on actual receptor affinities but also on other, less well predictable factors such as drug distribution and metabolism.

Interestingly, several snake toxins have been identified that display an unprecedented degree of mAChR subtype selectivity [16, 17]. For example, MT7 (m1-toxin 1) and MT3 (m4-toxin) are highly selective antagonists for M_1 and M_4 mAChRs, respectively [16, 17]. The binding of these polypeptide ligands appears to involve interactions with less well conserved amino acids present on the extracellular surface of the receptors [17].

5.4.3 Allosteric Ligands

The binding of muscarinic ligands to the classical (“orthosteric”) binding site can be modulated by so-called allosteric ligands which interact with a secondary (allosteric) site [29, 30]. In fact, the mAChRs are generally used as a model system to illustrate the molecular mechanisms by which GPCR activity can be modulated by allosteric drugs [30]. The first allosteric muscarinic ligands to be identified were gallamine and other neuromuscular blocking drugs, including alcuronium and pancuronium. However, during the past decade, many structurally diverse ligands have been identified that appear to bind to sites different from the classical mAChR binding site [29, 30].

All mAChR subtypes have been shown to be susceptible to allosteric modulation. However, the molecular nature of this modulation differs among the individual receptor subtypes and depends on the choice of orthosteric and allosteric ligands and their concentrations. The receptor binding site for allosteric muscarinic ligands is thought to be located just “above” the classical ligand binding pocket involving residues located in the extracellular loops and the outermost segments of different TM helices. The existence of an allosteric site on the mAChRs may lead to novel therapeutic strategies aimed at “turning off” or “tuning up” signaling through distinct mAChRs subtypes [29–31]. Most allosteric muscarinic ligands retard the dissociation of muscarinic radioligands bound to the orthosteric site [30]. Some allosteric ligands have been shown to display positive cooperativity with ACh or certain muscarinic antagonists at specific mAChR subtypes. The thiamine metabolite, thiochrome, for example, is an allosteric enhancer at M_4 receptors but has no effect on ACh binding to the remaining four mAChR subtypes [32].

Tränkle et al. [33] were able to develop dimethyl-W84 (N,N'-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2H-isoindol-2-yl)propyl]-N,N,N',N'-tetramethyl-1, 6-hexanediaminium diiodide) as the first allosteric muscarinic radioligand that can label an allosteric site on the M_2 mAChR. Interestingly, recent evidence suggests the existence of at least two allosteric sites with very different pharmacologies [34, 35].

Recent studies have also led to the identification of atypical muscarinic agonists that can activate M_1 receptors with rather high selectivity, most likely via interaction with an allosteric site. These agents include, for example, AC-42

[4-*n*-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; [36] and *N*-desmethylozapine [37]. The development of subtype-selective allosteric muscarinic ligands for therapeutic purposes, particularly allosteric enhancers, therefore appears a very attractive goal.

5.4.4 Muscarinic Drugs in Current Clinical Use

Muscarinic agonists and antagonists are in clinical use for the treatment of various pathophysiological conditions [4, 38]. Muscarinic agonists such as pilocarpine and aceclidine are applied locally to the eye for the treatment of glaucoma to reduce intraocular pressure. Moreover, muscarinic agonists such as carbachol or bethanechol are employed in certain cases of atonia of the stomach, bowel, or urinary bladder. The muscarinic agonists pilocarpine and cevimeline are used to stimulate salivary secretion under conditions where the function of the salivary glands is impaired [39]. Scopolamine, a muscarinic antagonist, is highly effective in preventing motion sickness. Centrally acting muscarinic antagonists (e.g., trihexyphenidyl, procyclidine, or biperiden) are useful for the treatment of Parkinson's disease or Parkinson-like symptoms caused by the administration of antipsychotic drugs, probably due to their ability to reduce excessive striatal muscarinic neurotransmission resulting from the lack of striatal dopamine. Muscarinic antagonists are also widely used in the treatment of disorders characterized by an increased tone or motility of the gastrointestinal and urogenital tracts and in the local therapy of obstructive pulmonary diseases, including chronic bronchitis and bronchial asthma. Additional uses of muscarinic antagonists are in ophthalmology to produce mydriasis and/or cycloplegia, the treatment of peptic ulcer disease (e.g., pirenzepine) and certain forms of cardiac arrhythmias, and their application as part of routine preoperative medication, primarily to reduce reflex bradycardia and excessive bronchial secretion [4, 38].

5.4.5 Muscarinic Drugs: Potential Future Clinical Applications

A major problem associated with the clinical use of classical muscarinic drugs is the rather common occurrence of side effects, primarily due to the limited receptor subtype selectivity of the muscarinic drugs used and the widespread distribution of the individual mAChRs. It is likely that the development of muscarinic agonists and antagonists that can interact with individual mAChRs with a high degree of selectivity will lead to novel muscarinic drugs with reduced side effects and increased efficacy [7, 8]. For example, centrally acting selective M₁ receptor agonists may become useful in the treatment of Alzheimer's disease, and selective M₃ receptor antagonists are predicted to produce fewer side effects in the treatment of smooth muscle disorders, including urinary urge incontinence, irritable bowel syndrome, and chronic obstructive pulmonary disease [7, 8]. The application of subtype-selective muscarinic drugs may also be beneficial in the management of pain and in the treatment of schizophrenia [7, 8]. Recent studies with mAChR knockout (KO) mice have led to novel insights into the physiological and pathophysiological roles of the individual mAChR subtypes [40]. As a result, these studies have opened new avenues for the development of novel muscarinic drugs potentially useful in the treatment of a variety of pathophysiological conditions (see Section 5.14).

5.5 MECHANISMS INVOLVED IN mAChR ACTIVATION

The current view is that the inactive state of mAChRs and other GPCRs is maintained by specific interhelical interactions. Agonist binding to GPCRs is predicted to disrupt this interhelical network of interactions, replacing it with a new set of intramolecular contacts that stabilize the active receptor conformation(s). Site-directed mutagenesis studies with the M₁ mAChR have shown that the removal of several of the aromatic residues predicted to be in close proximity to the ACh ammonium head group leads to mutant receptors with greatly reduced G-protein coupling efficiencies [25, 27]. These findings are consistent with the concept that ACh binding leads to a tightening of the charge-stabilized aromatic cage around the ion pair formed by the quaternary head group of ACh and the conserved TM III Asp residue [25, 27].

The molecular nature of the ACh-induced conformational changes is not well understood at present. In any case, the conformational changes predicted to occur close to the ligand binding pocket need to be transmitted to the intracellular surface of the mAChR protein, enabling the receptor to productively interact with specific classes of heterotrimeric G proteins. Recent *in situ* disulfide crosslinking studies have shown, for example, that agonist activation of the M₃ mAChR leads to pronounced structural changes on the intracellular surface of the receptor protein [26, 41]. Receptor activation is predicted to be associated with conformational changes that increase the proximity of the cytoplasmic ends of TM VI and TM V [41] and of TM VII and TM I [26], respectively. These movements also appear to include major rotational movements of the cytoplasmic ends of TMs VI and VII [26, 41]. These agonist-induced structural changes are predicted to expose previously inaccessible residues or surfaces, for example on TMs III, VI, and VII, to G-protein heterotrimers, ultimately triggering productive receptor–G protein coupling.

During the past decade, several point mutations located in different receptor domains have been identified that render specific mAChR subtypes constitutively active [42–46], a phenomenon that has been observed with many other classes of GPCRs. It is generally assumed that the majority of these mutations interfere with the proper packing of the TM receptor core, thus mimicking, at least partially, the agonist-induced “opening” of the intracellular receptor surface. It should also be noted that constitutive receptor activity (activity in the absence of agonists) has also been observed with mAChRs expressed in native tissues, including cardiac membranes [47]. All classical muscarinic antagonists that have been tested so far are able to act as inverse agonists, being able to suppress constitutive signaling by wild-type (WT) or mutant mAChRs [47, 48].

Interestingly, a recent study analyzing mAChR–G protein interactions by the use of [³⁵S]-GTPγS (Guanosine-5'-O-(3-[³⁵S]-thio)-triphosphate) binding assays suggests that the specific G-protein coupling profile of a given mAChR may also depend on the chemical nature of the activating ligand [49]. This finding is consistent with observations made with many other classes of GPCRs, suggesting that structurally different agonists may stabilize slightly different active receptor conformations [50].

5.6 G-PROTEIN COUPLING PROPERTIES OF mAChRs

Based on their G-protein coupling properties, the M₁–M₅ mAChRs can be subdivided into two major functional subclasses [2, 3, 51]. The M₁, M₃, and M₅

mAChRs are preferentially coupled to G proteins of the $G_{q/11}$ family, which mediate the activation of different isoforms of phospholipase C β (PLC β), resulting in the breakdown of phosphatidyl inositol (PI) and the generation of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP $_3$). DAG activates protein kinase C (PKC), and IP $_3$ induces the release of calcium from intracellular stores. The resulting increase in intracellular calcium levels ($[Ca^{2+}]_i$) regulates the activity of many ion channels and enzymes including the activation of calmodulin-dependent protein kinases, adenylyl cyclases, and phosphodiesterases. The activated $G_{q/11}$ subunits can stimulate the activity of all members of the PLC β family (PLC β 1–4 [52]).

In contrast, the M_2 and M_4 mAChRs are selectively linked to G proteins of the $G_{i/o}$ class (these G proteins can be inactivated by treatment with pertussis toxin), which, at a biochemical level, inhibit the accumulation of intracellular cyclic adenosine monophosphate (cAMP) via inhibition of adenylyl cyclase [2, 3, 51]. Activation of the M_2 and M_4 mAChRs can also lead to the activation of the PLC β 2 and PLC β 3 isoforms [52, 53]. This response has been shown to be mediated by free $G\beta\gamma$ subunits released after activation of G proteins of the $G_{i/o}$ family [52, 53].

It should be noted, however, that the G-protein coupling preference of the individual mAChR subtypes is relative rather than absolute and depends on a number of factors including receptor and G-protein levels and cell type under investigation [2, 51].

The regulation of adenylyl cyclase activity by different mAChRs is particularly complex [51, 54]. One major complicating factor is the existence of at least nine adenylyl cyclase isoforms which differ in their regulation by different G-protein subunits and second messengers [55]. Activated $G_{\alpha_{i/o}}$ subunits generated by activation of M_2 and M_4 mAChRs can directly inhibit several adenylyl cyclase isoforms [55]. However, in certain cells or tissues, activation of G_q -coupled mAChRs may also result in reduced intracellular cAMP levels [51]. This response may involve the stimulation of a calcium–calmodulin sensitive phosphodiesterase activated by mAChR-mediated increases in $[Ca^{2+}]_i$ [56]. Moreover, when expressed at high levels in certain cell types, both G_q - and G_i -linked mAChRs can also couple to G_s to stimulate adenylyl cyclase activity [51, 54]. G_q -coupled mAChRs have also been shown to increase intracellular cAMP levels via activation of calcium–calmodulin-sensitive adenylyl cyclases [57]. Moreover, Olanas et al. [58] demonstrated that G_i -coupled mAChRs expressed in the rat olfactory bulb can facilitate increases in intracellular cAMP levels via $G\beta\gamma$ -dependent activation of adenylyl cyclases II and IV.

Accumulating evidence also suggests that the G-protein coupling profiles of the individual members of the two functional classes of mAChRs may not be identical [2, 22, 51]. Migeon et al. [59], for example, demonstrated that the M_2 and M_4 mAChRs, when transiently expressed in JEG-3 cells, couple to inhibition of adenylyl cyclase by overlapping but different G-protein $\alpha_{i/o}$ subunits. Similarly, studies with stably transfected CHO cell lines showed that the M_1 receptor coupled to stimulation of adenylyl cyclase with significantly greater efficiency than the M_3 receptor subtype [60].

The selectivity of mAChR–G protein interactions can also be modulated by the type of G-protein $\beta\gamma$ complexes expressed within a cell [61], providing an additional layer of functional complexity. Studies with antisense oligonucleotides demonstrated,

for example, that specific $G\beta$ ($\beta 3$) and $G\gamma$ ($\gamma 4$) subunits are required to allow M_4 receptor-mediated inhibition of voltage-sensitive calcium channels in GH3 cells via coupling to $G\alpha_{o1}$ [61]. Similarly, studies with M_2 mAChR-expressing Sf9 insect cells showed that the M_2 receptor could activate G_o heterotrimers with different efficacies, depending on the molecular identity of the β and/or γ subunit contained within the G-protein heterotrimer [62, 63].

5.7 STRUCTURAL BASIS OF SELECTIVITY OF mAChR-G PROTEIN INTERACTIONS

Following agonist binding, mAChRs, like other GPCRs, undergo conformational changes that allow residues located on the intracellular receptor surface to productively interact with heterotrimeric G proteins. Studies with chimeric mAChRs, complemented by site-directed mutagenesis studies, have identified the key receptor domains and amino acids that determine the G-protein coupling profile of the individual mAChRs. This work has been reviewed in detail previously [2, 22]. The mAChR regions known to be critically involved in determining the selectivity of receptor-G protein interactions are the i2 domain and the membrane-proximal portions of the i3 loop. Several studies have shown that these regions act in a cooperative fashion to determine which set of G proteins are activated by a specific mAChR [2, 22]. Fig 5.1 highlights the specific residues in the rat M_3 muscarinic receptor that are critically involved in the selective coupling of this receptor subtype to G proteins of the G_q family. We previously proposed that the functionally important residues at the N- and the C-terminus of the i3 loop form two adjacent hydrophobic patches that project into the interior of the receptor protein [2, 21, 22]. These two hydrophobic surfaces, together with several hydrophilic residues in the i2 loop, are thought to form a G-protein binding pocket, representing the primary structural determinant of the selectivity of mAChR-G protein interactions. According to this model, this functionally critical receptor surface cannot be properly recognized by the G protein in the resting state of the receptor. However, the agonist-induced conformational changes are thought to lead to an opening of the intracellular receptor surface, thus allowing the G protein to make productive contacts with key residues on the receptor protein ([2, 21, 22]; see Section 5.5 for more details).

The molecular architecture of the receptor-G protein complex still remains only poorly defined. Many studies have shown that the C-terminus of the G-protein α subunits plays a key role in determining the selectivity of receptor-G protein interactions [64–66]. To identify the site on the M_2 mAChR that can interact with the C-terminus of $\alpha_{i/o}$ subunits, Liu et al. [65] took advantage of the observation that the M_2 mAChR did not recognize wild-type α_q but was able to efficiently couple to mutant α_q subunits in which the last five amino acids of α_q were replaced with the corresponding α_i or α_o sequences. The outcome of a series of loss- and gain-of-function mutagenesis studies strongly suggested that a four-amino-acid motif ('VTIL', corresponding to Val385, Thr386, Ile389, and Leu390 in the human M_2 mAChR), located at the i3 loop-TM VI junction, can selectively recognize the C-terminus of $\alpha_{i/o}$ subunits [65]. This agonist-induced interaction is predicted to play a key role in productive receptor-G protein coupling.

To identify the amino acids on the rat M_3 mAChR that can interact with the C-terminus of α_q , Kostenis et al. [66] again used a coexpression strategy involving the use of M_2/M_3 hybrid mAChRs and chimeric G-protein α subunits. This analysis showed that the AALS motif (Ala488, Ala489, Leu492, and Ser493 in the rat M_3 mAChR sequence; Fig. 5.1), which corresponds to the VTIL sequence in the M_2 mAChR, plays an important role in recognizing the C-terminal amino acids of α_q . However, these studies also suggested that amino acids within the i2 loop and the N-terminal portion of the i3 domain of the M_3 mAChR may also be able to contact the C-terminus of α_q . One possible explanation for this observation is that the C-terminal portion of α_q can contact a receptor surface composed of amino acids located on different intracellular receptor regions. This notion is also supported by loss-of-function studies [67] examining the ability of a C-terminal α_t (transducin) peptide to bind to WT and mutant versions of rhodopsin.

Although it is clear that the C-terminus as well as other regions of the G-protein α subunits play a predominant functional role in receptor–G protein coupling, considerable evidence suggests that G-protein $\beta\gamma$ complexes can also modulate the selectivity of receptor–G protein interactions, presumably by directly contacting the receptor protein [61, 68].

5.8 MODULATION OF mAChR–G PROTEIN COUPLING BY RGS PROTEINS

The efficiency of mAChR–G protein interactions is modulated by the activity of a class of proteins referred to regulators of G-protein signaling (RGS proteins [69]). RGS proteins act as negative regulators of G-protein signaling by serving as GTPase-activating proteins for $G\alpha$ subunits, thereby accelerating G-protein inactivation [69]. Several recent studies have shown that signaling through distinct mAChR subtypes is reduced by the activity of specific classes of RGS proteins (see, e.g., [70–75]). Interestingly, Bernstein et al. [71] recently reported that RGS2 can bind simultaneously to the i3 loop of the M_1 mAChR and activated $G\alpha_q$. Moreover, the ability of RGS2 to inhibit M_1 receptor-mediated activation of $PLC\beta$ correlated well with its ability to bind to the i3 loop of the M_1 receptor. These data support the novel concept that the specificity of RGS–G protein interactions *in vivo* is modulated by selective recognition of specific members of the RGS family by a given GPCR.

5.9 SIGNALING PATHWAYS MODULATED BY mAChR ACTIVATION

As already noted in Section 5.6, mAChRs regulate, either directly or indirectly, the activity of a very large number of intracellular signaling pathways and molecules. These include, besides the pathways already discussed earlier in this chapter, phospholipases A_2 and D, small G proteins of the rac and rho family, protein tyrosine kinases such as Bruton's tyrosine kinase [76] and PYK2 (Proline-rich tyrosine kinase 2; [77]), and different NO synthase isoforms [54, 78]. A detailed description of these pathways is beyond the scope of this chapter (for a recent review, see [78]). The following section will summarize a series of studies dealing with the role of mAChRs

in regulating different mitogen-activated protein kinase (MAPK) pathways. These kinases play key roles in many important physiological functions, including, for example, regulation of gene transcription, cellular growth and differentiation, and synaptic plasticity [78].

5.10 MODULATION OF MAPK PATHWAYS

A wealth of evidence indicates that agonist stimulation of mAChRs, as well as of many other GPCRs, can activate various MAPKs [51, 78, 79]. mAChR-mediated activation of MAPK can occur via a variety of cellular mechanisms, depending on the type of mAChR and cell studied. MAPK activation can be mediated by both activated $G\alpha$ subunits and $G\beta\gamma$ complexes [51, 78, 79].

The MAPK family consists of four major subgroups, the extracellular signal-regulated kinases (ERK1 and 2), the stress-activated protein kinases or c-Jun N-terminal kinases (SAPKs/JNKs), p38 kinases, and ERK5 [79].

ERK1 (p44 MAPK) and ERK2 (p42 MAPK) can be activated by stimulation of either of the five mAChR subtypes [78, 80]. Depending on the investigated cell type and the specific experimental conditions used, M_1 , M_3 , and/or M_5 mAChR-mediated ERK activation can occur via PKC-dependent and PKC-independent mechanisms [78]. M_2 and M_4 mAChR-mediated ERK activation has been shown to be mediated by free $G\beta\gamma$ complexes. Lopez-Ilasaca et al. [81], for example, demonstrated that stimulation of M_2 mAChRs transiently expressed in COS-7 cells leads to $G\beta\gamma$ -dependent activation of phosphoinositide 3-kinase γ (PI3K γ), which in turn triggers the activation of the Shc–Grb2–Sos–Ras pathway, resulting in increased MAPK activity. However, another study showed that MAPK stimulation can also be mediated by activated $G\alpha_i$, generated by stimulation of M_2 receptors expressed by HEK-293T cells [82]. Additional studies suggested that MAPK stimulation is mediated by a $G\alpha_i$ -dependent reduction in the levels of the active form of Rap1, an inhibitor of Ras function, involving the activation of a novel Rap1 GTPase-activating protein [82].

Several studies have shown that activation of various mAChR subtypes can also lead to the activation of JNK, p38 kinases, and ERK5 in different cell types or tissues. As is the case for mAChR-dependent activation of ERK1 and 2, multiple cellular pathways appear to participate in mAChR-mediated stimulation of these MAPKs [78, 79].

Activation of several GPCRs, including the M_1 mAChR, can also lead to the transactivation of cell surface growth factor receptors known to be linked to MAPK activation. For example, studies with transfected Rat-1 and COS-7 cells demonstrated that M_1 mAChR activation leads to the transactivation of the EGF (epidermal growth factor) receptor [83]. This transactivation process appears to require the activity of a metalloproteinase which releases an EGF-like factor capable of activating the EGF receptor [83]. In contrast, an inhibition of EGF receptor-mediated Raf and ERK activation was observed after stimulation of M_1 mAChRs expressed in Rat-1a fibroblasts, probably due to M_1 receptor-mediated activation of protein kinase A (PKA) following stimulation of a calcium/calmodulin-sensitive adenylyl cyclase isoform [84].

5.11 ION CHANNELS

Agonist stimulation of mAChRs modulates the activity of a large number of ion channels, by either direct or indirect mechanisms, resulting in “excitatory” or “inhibitory” effects. This subject has been the topic of several excellent reviews [9, 78, 85, 86]. The excitatory effects primarily result from the closure of different types of potassium channels but may also involve the opening of cation channels, at least in some cases. Most of the inhibitory effects are mediated by the opening of potassium channels and the closure of voltage-gated calcium channels.

5.11.1 Closure of Potassium Channels

Activation of M_1 and M_3 mAChRs has been shown to mediate the inhibition of K_M channels (M current [85]). For example, Hamilton et al. [87] demonstrated that muscarinic agonist-mediated inhibition of the M current was abolished in sympathetic ganglion neurons derived from M_1 receptor KO mice. Suppression of the M current, a voltage-dependent potassium current found in various neuronal tissues including sympathetic ganglia and cortical and hippocampal neurons, leads to an increase in neuronal excitability and facilitates repetitive spike discharges [85]. Structurally, neuronal-type M channels are predicted to be composed of a heteromeric array of KCNQ2 and KCNQ3 potassium channels [88, 89]. Suh and Hille [90] recently showed that M_1 mAChR-mediated activation of PLC initiates M current modulation and that channel recovery requires ATP and PI 4-kinase, suggesting that the breakdown of PI 4,5-bisphosphate (PIP_2) represents a key factor in M current modulation.

Agonist stimulation of mAChRs also leads to the closure of several other potassium channels, including the “small” calcium-activated potassium channel (K_{Ca} [85]). This channel is widely distributed in peripheral and central neurons and is known to generate long post-spike after-hyperpolarizations (APHs). ACh-induced inhibition of this channel represents a major component of mAChR-mediated excitation of central neurons [85]. At present, it is not entirely clear which specific mAChRs are involved in mediating this activity.

In addition, activation of mAChRs also leads to the inhibition of several other classes of neuronal potassium channels/currents, including “inward-rectifier” potassium channels (K_{IR}), fast-gating M-like currents, resting “leak” potassium currents, and several cloned potassium channels including Kv1.2. In most cases, M_1 and/or M_3 mAChRs play a predominant role in mediating the inhibition of these channels/currents [85].

5.11.2 Opening of Potassium Channels

Activation of M_2 or M_4 mAChRs can lead to the opening of G-protein-regulated inward-rectifier potassium (GIRK) channels. Neuronal GIRK channels are thought to be involved in regulating neuronal excitability by contributing to the resting potential [91]. Many of the key studies on GIRK channel regulation were performed on the cardiac K_{ACh} channel, which is composed of GIRK1 and GIRK4 subunits. This channel is directly activated by $G\beta\gamma$ subunits released following M_2 receptor-mediated activation of G proteins of the G_i family [91]. It is likely that neuronal

GIRK channels are activated by a similar mechanism [78, 85, 91]. Studies with GIRK4 KO mice have shown that the activation of cardiac K_{ACh} channels plays an important role in ACh- and adenosine-mediated bradycardia in vivo [92]. Interestingly, several studies suggest that G_q -coupled mAChRs may be able to inhibit GIRK channel activity [78].

5.11.3 Modulation of Calcium Channels

As discussed in Section 5.6, activation of the G_q -coupled mAChRs leads to pronounced increases in $[Ca^{2+}]_i$, primarily via $PLC\beta$ -mediated generation of IP_3 . However, mAChRs can also modulate $[Ca^{2+}]_i$ by regulating the activity of voltage-operated calcium channels (VOCCs) that open when the cell membrane is depolarized. The VOCC family is classified into L, N, P/Q, R, and T subtypes, and there is evidence that mAChRs can modulate the activity of all of these channels [78].

Activation of M_2 and M_4 mAChRs results in the inhibition of VOCCs [85]. This activity is probably mediated by free $G\beta\gamma$ subunits released following the activation of G_i -type G proteins and may be responsible for the well-known autoinhibitory effects of ACh on its own release observed in many neuronal tissues [85].

In sympathetic ganglion neurons, muscarinic agonists inhibit N- and L-type calcium channels through two distinct G-protein-mediated pathways [93]. Studies with mAChR KO mice showed that the fast and voltage-dependent pathway is mediated by M_2 receptors while the slow and voltage-independent pathway is mediated by M_1 receptors [93].

Interestingly, several studies suggest that the activity of several VOCC subtypes can also be enhanced following muscarinic stimulation, at least in certain tissues or cell types and under certain experimental conditions (for a detailed review of these studies, see [78]).

5.11.4 Nonselective Cation Channels

Muscarinic stimulation can also result in the opening of nonselective cation channels in several peripheral and central tissues or cell types. For example, activation of M_2 receptors is known to lead to the opening of a nonselective cation channel in smooth muscle cells, a response predicted to play a role in smooth muscle contraction [94, 95]. The activation of coexpressed smooth muscle M_3 receptors has been shown to play a permissive role in this activity [95, 96]. Muscarinic agonists can also induce the activation of nonselective cation channels in different regions of the brain [78]. Analysis of a series of mAChR KO mice showed that muscarine-induced hippocampal γ oscillations were dependent on M_1 receptor-mediated depolarization of hippocampal CA3 pyramidal neurons, involving the activation of the mixed sodium-potassium current I_h and the calcium-dependent nonspecific cation current I_{cat} [97].

5.11.5 Chloride Channels

Several reports suggest that activation of mAChRs can trigger the opening of chloride channels in various tissues. In most cases, this activity is dependent on the primary increase in $[Ca^{2+}]_i$ [78]. In salivary gland acinar cells, for example,

ACh-mediated activation of G_q -coupled mAChRs (M_1 and M_3) is predicted to stimulate fluid secretion via activation of a calcium-gated chloride channel [98].

Marsh et al. [99] identified a delayed calcium-dependent chloride channel in sympathetic ganglion cells which is induced by the synergistic action of calcium and DAG. These authors demonstrated that the increase in these two second messengers was mediated by the simultaneous activation of nicotinic AChRs (leading to a rise in $[Ca^{2+}]_i$) and mAChRs (leading to increased DAG levels), indicative of a novel form of AChR synergy.

5.12 REGULATION OF mAChR ACTIVITY

Prolonged agonist exposure of mAChRs and other GPCRs leads to an attenuation of receptor-mediated responses referred to as receptor desensitization. Molecular events closely associated with receptor desensitization are “uncoupling,” receptor internalization (sequestration), and receptor downregulation. Receptor phosphorylation events play central roles in mediating many of these processes. Several different kinases have been implicated in mAChR phosphorylation, including various GPCR kinases (GRKs), casein kinase 1 α (CK1 α), and PKC [100, 101]. The phosphorylation of mAChRs has been shown to occur on Ser and Thr residues located within the i3 loop and the cytoplasmic tail [2, 100, 101].

5.12.1 Receptor Uncoupling

The earliest phase of receptor desensitization involves receptor uncoupling in which receptors become functionally uncoupled from their G proteins. Uncoupling occurs with a very rapid time course (seconds to minutes) and may serve to quickly attenuate agonist-induced signaling to prevent overstimulation. A vast body of evidence indicates that receptor phosphorylation plays a key role in mediating this process (see Section 5.12.4).

5.12.2 Receptor Internalization

A second step associated with receptor desensitization which occurs within minutes after agonist addition is receptor internalization [100–102]. This process is defined as removal of receptors from the cell surface without concomitant loss of total cellular receptor binding sites. As has been shown for many other classes of GPCRs, agonist-induced phosphorylation of mAChRs (see Section 5.12.4) is predicted to facilitate receptor internalization [101, 103]. However, several lines of evidence suggest that agonist-induced mAChR internalization does not require the activation of heterotrimeric G proteins [101]. Moreover, the role of specific GRKs in mediating mAChR internalization depends on the specific receptor subtype and cell type studied.

Considerable evidence indicates that mAChRs, like other GPCRs, can be internalized via multiple mechanisms [100, 101]. The GPCR internalization pathway that is understood best is GRK and arrestin dependent. In this pathway, the binding of arrestin 2 and 3 (see Section 5.12.4.2) to GRK-phosphorylated receptors leads to receptor endocytosis mediated by arrestin binding to clathrin present in clathrin-coated pits [104, 105]. This internalization pathway requires the presence of the

GTPase dynamin, which is required for pinching off receptor-containing vesicles from the plasma membrane [104, 105].

Studies with transfected HEK cells have shown that M_1 , M_3 , and M_4 mAChRs internalize in an arrestin-dependent fashion via clathrin-coated vesicles [101]. However, in the same cell type, M_2 mAChR internalization occurs through an arrestin-independent mechanism [101], suggesting that phosphorylated M_2 mAChRs may be able to interact with other cellular proteins to enter an internalization pathway that is clathrin independent. Interestingly, studies involving the use of dominant-negative mutant forms of dynamin strongly suggest that dynamin plays a role in the internalization pathway used by the M_1 , M_3 , and M_4 mAChRs as well as the M_2 mAChR [101].

Biochemical studies with M_2 mAChRs expressed in cardiac myocytes have shown that M_2 receptors undergo agonist-dependent trafficking to caveolae, small flask-shaped invaginations of the plasma membrane [106]. In a secondary step, these receptor-containing caveolae are predicted to be pinched off from the plasma membrane, leading to the internalization of a significant portion of M_2 receptors [107].

The structural elements involved in mAChR internalization have been studied by mutational analysis of several mAChR subtypes [2, 101]. For example, site-directed mutagenesis studies with the M_1 mAChR have shown that most mutations that interfere with productive receptor–G protein coupling also impair M_1 mAChR internalization [108]. However, several mutant M_1 receptors were identified that were defective in G-protein coupling but were able to internalize normally [108], indicating that G-protein activation is not an absolute requirement for receptor internalization to occur. Taken together, these findings support the concept that the receptor domains involved in mAChR internalization overlap but are not identical with those critical for receptor–G protein coupling.

5.12.3 Receptor Downregulation

In contrast to receptor internalization, receptor downregulation can be observed in many cell types upon prolonged agonist exposure (hours) and is characterized by a decrease in the total number of cellular receptors [102]. Return of receptor levels to control levels requires *de novo* protein synthesis. The role of receptor internalization in GPCR downregulation is incompletely understood at present and seems to depend on the specific GPCR studied and the cellular background in which it is expressed.

In many, but not all, cases, receptor-mediated G-protein activation appears to be required for receptor downregulation to occur. Several lines of evidence suggest that the early stages of receptor downregulation are mainly due to an increased rate in receptor degradation [107], while later stages may involve decreases in receptor mRNA levels (see, e.g., [109]).

Mutational analysis of the mouse M_1 mAChR expressed in Y1 adrenal carcinoma cells [110] and the human M_2 mAChR expressed in CHO cells [111] suggests that sequences within the i3 loop play a key role in mAChR downregulation. Using stably transfected CHO cells, Shockley et al. [112] identified several point mutations in the i2 and i3 loops of the human M_1 receptor that interfered with receptor downregulation but not internalization. Moreover, studies with stably transfected CHO cells showed that three Thr residues present in the C-terminal tail domain of the human M_3 mAChR are required for proper receptor downregulation (corresponding

to residues 549, 552, and 553 in Fig. 5.1 [113]). Similarly, Goldman and Nathanson [114] found that the lone Tyr residue in the C-terminal region of the porcine M₂ mAChR (Tyr459) heterologously expressed in JEG-3 cells is involved in receptor downregulation but not in receptor internalization and G-protein coupling.

As summarized in the previous paragraph, many of the analyzed mutant mAChRs that showed deficits in downregulation did not show significant impairments in short-term receptor internalization. This observation clearly indicates that receptor internalization is not an absolute requirement for mAChR downregulation to occur.

5.12.4 Receptor Phosphorylation

Characteristically, the second-messenger-dependent protein kinases, such as PKC or PKA, are able to phosphorylate GPCRs even in the absence of agonist, suggesting that they may play a role in heterologous receptor desensitization. On the other hand, GRKs can only recognize the agonist-activated forms of GPCRs and are thought to play key roles in short-term homologous receptor desensitization.

5.12.4.1 Protein Kinase C. PKC is capable of phosphorylating mAChRs and other GPCRs in an agonist-independent fashion. It has been shown that both the M₁ and the M₃ mAChRs are phosphorylated by PKC in an agonist-independent fashion both in vitro and in vivo [101]. For example, Haga et al. [115] demonstrated that the M₁ mAChR is phosphorylated by PKC on Thr and Ser residues located within the cytoplasmic tail and the C-terminal segment of the i3 loop. Importantly, activation of PKC by phorbol esters led to impaired mAChR signaling in a number of different cell types predicted to express M₁ and/or M₃ mAChR [101]. Since stimulation of M₁ and M₃ mAChRs results in the generation of the potent PKC activator DAG, it is likely that PKC-mediated phosphorylation of these receptors serves as a negative-feedback mechanism to dampen M₁ and M₃ receptor signaling.

5.12.4.2 GPCR Kinases. GRKs play fundamental roles in the homologous desensitization of mAChRs and other GPCRs [105, 116]. In contrast to the second-messenger-activated protein kinases, GRKs only phosphorylate the agonist-activated forms of GPCRs. In mammals, the GRK family consists of seven distinct members, GRK1–7 [105, 116]. Whereas GRK1 and GRK7 are only expressed in the retina, GRK2, 3, 5, and 6 are expressed ubiquitously. GRK4 is preferentially expressed in the testes and brain. The GRK-phosphorylated receptors are able to bind to a group of proteins referred to as arrestins which prevent receptor–G protein signaling by blocking the intracellular receptor surface [104, 117]. The arrestin family consists of the two visual arrestins and arrestin 2 (β -arrestin 1) and arrestin 3 (β -arrestin 2). The latter two arrestins are expressed ubiquitously [104, 117].

In vitro and in vivo studies have shown that agonist-activated M₁–M₄ mAChRs are phosphorylated by different members of the GRK family (GRK2, 3, 5, and 6 [100, 101, 103]). Many of these studies used strategies that involved the over-expression of WT GRKs or mutant GRKs endowed with dominant-negative activity. However, it is often difficult to conclude from these studies which specific GRKs regulate the function of individual mAChRs in specific tissues or cell types in vivo [100]. Recently, novel experimental strategies including the use of GRK KO mice and

antisense approaches have been applied to overcome the difficulties associated with the use of overexpression approaches.

The M₁ and M₃ mAChRs have been shown to be phosphorylated by purified GRK2 in an agonist- and Gβγ-dependent fashion ([115, 118]; note that free Gβγ subunits are required for trafficking GRK2 and 3 to the plasma membrane). The major GRK2 phosphorylation sites of the human M₁ mAChR are predicted to be located within a segment contained within the i3 loop (amino acids 276–303 [115]). Similarly, studies with GST fusion proteins suggest that GRK2 phosphorylates the rat M₃ mAChR on multiple Ser residues located within the i3 loop (³³¹SSS³³³ and ³⁴⁸SASS³⁵¹ [119]). These authors also identified a putative Gβγ binding site in the rat M₃ mAChR (amino acids 289–330) that is located immediately N-terminal of the GRK2 target sequence.

Recent studies strongly suggest that endogenous GRK2 plays a key role in mediating agonist-induced desensitization of M₁ mAChRs expressed by rat hippocampal neurons [120]. Interestingly, the regulatory activity of GRK2 appears to be mediated by both phosphorylation-dependent and phosphorylation-independent mechanisms [120].

Studies with transfected mammalian cells have shown that the human M₃ mAChR is also subject to agonist-dependent phosphorylation by CK1α [121–123]. Consistent with this notion, coexpression of a catalytically inactive mutant of CK1α strongly inhibited agonist-induced M₃ mAChR phosphorylation [121]. Analysis of an M₃ receptor deletion mutant lacking amino acids 370–425 suggested that the putative CK1α phosphorylation sites are likely to be located within a segment of the i3 loop that is located C-terminal of the region containing the putative GRK2 phosphorylation sites (see above [122]). Following agonist stimulation, this M₃ receptor deletion mutant retained the ability to efficiently stimulate PLC but was significantly impaired in its ability to stimulate the ERK1/2 pathway [122]. These observations suggest that CK1α-mediated phosphorylation of specific intracellular M₃ receptor residues is required for the efficient activation of the ERK1/2 pathway, perhaps by allowing the recruitment of site-specific adaptor proteins [122, 123].

Willets et al. [124, 125] recently demonstrated that agonist-induced phosphorylation and desensitization of M₃ mAChRs endogenously expressed by human SH-SY5Y cells is mediated primarily by GRK6.

Studies with GRK3-deficient mice indicate that M₃ receptor signaling may be regulated by GRK3 phosphorylation *in vivo*, at least in certain tissues. Recently, Fisher et al. [126] demonstrated that methacholine-induced bronchoconstriction is completely abolished in M₃ mAChR KO mice, suggesting that mAChR-mediated airway constriction is mediated by the M₃ receptor subtype *in vivo*. Interestingly, methacholine-induced bronchoconstriction was significantly enhanced in GRK3-deficient mice [127], suggesting that airway M₃ mAChRs may be a target of GRK3 *in vivo*.

Many studies have implicated GRKs (GRK2, 3, and 5) in the phosphorylation and desensitization of the M₂ receptor subtype [100, 101, 103]. Strikingly, studies with transfected HEK293 cells showed that mutant human M₂ receptors lacking the central portion of the i3 loop (amino acids 282–323 [128]) or containing simultaneous mutational modifications of two clusters of Thr and Ser residues located within this sequence [129] were no longer subject to agonist-dependent phosphorylation and desensitization.

Studies with GRK5-deficient mice demonstrated that many of the central actions of oxotremorine, a non-subtype-selective muscarinic agonist, were enhanced or prolonged in the absence of GRK5 [130]. These responses included oxotremorine-induced hypothermia, tremor, hypolocomotion, and analgesia. Phenotypical analysis of M₂ receptor KO mice showed that these responses are mediated exclusively or predominantly by the M₂ receptor subtype [131]. These studies therefore suggest that GRK5-mediated phosphorylation leads to the desensitization of central M₂ receptors *in vivo*. A recent study using GRK5-deficient mice also indicated that the activity of bronchial M₂ mAChRs may also be regulated by GRK5 [132].

Relatively little is known about the potential roles of GRKs in regulating the functions of the M₄ and M₅ mAChR subtypes. Holroyd et al. [133] presented data suggesting that GRK2 plays a key role in mediating internalization and desensitization of M₄ mAChRs endogenously expressed by NG108-15 cells. Studies with COS-7 cells transiently expressing the human M₅ mAChR showed that agonist-induced M₅ receptor internalization was facilitated by coexpression of GRK2 [134]. However, it remains to be shown whether the M₅ receptor is indeed a direct target for GRK2-mediated phosphorylation.

5.12.5 Mechanisms Involved in mAChR Resensitization

Accumulating evidence suggests that receptor internalization and recycling to the cell surface play key roles in restoring normal signaling function to desensitized GPCRs [104]. However, several studies have shown that the precise role of receptor internalization and recycling for receptor resensitization is critically dependent on the specific GPCR being analyzed and the type of cell by which it is expressed. This concept is highlighted by the outcome of studies carried out with M₃ or M₄ mAChRs expressed in different cellular backgrounds. Whereas receptor internalization and recycling appeared to be required for receptor resensitization in some cases, these steps did not prove to be essential for the receptor resensitization in other cell types (reviewed by [101]).

5.13 mAChR DIMERIZATION

Accumulating evidence suggests that GPCRs, including different members of the mAChR family, exist in the plasma membrane as dimeric or oligomeric complexes [135–137]. Several studies demonstrated that GPCR dimerization occurs in the endoplasmic reticulum and that dimeric GPCRs, following their transport to the cell surface, may represent the basic functional receptor unit able to productively interact with heterotrimer G proteins [138].

Early radioligand-binding studies indicated that orthosteric muscarinic ligands could bind to mAChRs in a cooperative fashion, a behavior that could be explained by the existence of mAChR dimers or oligomers (see [139] and references therein). Maggio et al. [140] carried out a series of cotransfection experiments involving two M₃ muscarinic/ α_2C -adrenergic hybrid receptors none of which was able to bind muscarinic or adrenergic ligands and to couple to G_q when expressed alone in COS-7 cells. However, coexpression of the two hybrid receptors resulted in the appearance of a small but significant number of muscarinic and adrenergic binding sites, and

incubation of cotransfected cells with the muscarinic agonist carbachol led to a pronounced increase in the breakdown of PI lipids. Similar results were obtained in studies in which two different coupling-deficient mutant M_3 receptors were coexpressed in COS-7 cells [135, 140]. These findings provided strong support for the concept that M_3 mAChRs can exist in dimeric or oligomeric complexes.

Moreover, co-immunoprecipitation studies using different versions of the M_3 [141] or M_2 [142, 143] mAChRs carrying different epitope tags also confirmed the existence of mAChR homodimers/oligomers. Studies with M_2 mAChRs expressed in Sf9 insect cells suggest that M_2 receptor aggregates are at least trimeric [142, 143]. Immunoprecipitation and immunoblotting studies demonstrated that M_3 receptor dimers are expressed on the cell surface and can bind muscarinic ligands [141]. Their formation seems to involve both noncovalent as well as disulfide bond interactions between receptor monomers [141]. In transfected COS-7 cells, M_3 receptor dimerization is insensitive to regulation by ligands [141]. Similar results have been obtained with M_2 mAChRs expressed in Sf9 insect cells [142].

Based on coexpression studies analyzing various mutant M_2 and M_3 mAChRs, Maggio et al. [144] suggested the existence of M_2/M_3 receptor heterodimers endowed with a novel ligand binding pharmacology (see, however, [141]). It remains to be seen whether such mAChR heterodimers are of physiological relevance. Another set of coexpression studies involving the use of different M_3 mutant receptors as well as of M_3 muscarinic/ α_{2C} -adrenergic hybrid receptors demonstrated that muscarinic activation of the ERK1/2 pathway required the simultaneous activation of two receptor monomers contained within a dimeric receptor assembly [145]. In addition, Novi et al. [146] recently presented data suggesting that M_3 receptor-mediated recruitment of β -arrestin 1 requires the simultaneous stimulation of the two monomeric units contained within a receptor dimer.

5.14 PHENOTYPICAL ANALYSIS OF mAChR-DEFICIENT MICE

Because of the lack of ligands endowed with a high degree of receptor subtype selectivity and the fact that most tissues or cell types express two or more mAChR subtypes, identification of the physiological and pathophysiological roles of the individual mAChR subtypes has proven a difficult task. To circumvent these difficulties, we and other laboratories applied gene-targeting techniques to generate mutant mouse lines deficient in each of the five mAChR genes (for recent reviews, see [40, 147, 148]). Mutant mice lacking M_1 , M_2 , M_3 , M_4 , or M_5 mAChRs are viable and fertile, appear generally healthy, and do not display any gross behavioral or morphological abnormalities. However, detailed phenotyping studies showed that each of the five mutant mouse lines displayed characteristic physiological, pharmacological, behavioral, biochemical, or neurochemical deficits. Disruption of one specific mAChR gene does not seem to have major effects on the expression levels of the remaining four mAChRs [40, 149, 150], an observation that greatly facilitates the interpretation of results of mouse phenotyping studies.

The following paragraphs will summarize the key findings that have emerged from the phenotypical analysis of M_1 – M_5 mAChR mutant mice. Homozygous M_1 – M_5 mAChR mutant mice will be referred to as $M1R^{-/-}$ mice, $M2R^{-/-}$ mice, and so on.

5.14.1 Functions Mediated by Central mAChRs

5.14.1.1 Learning and Memory. The M_1 mAChR is abundantly expressed in higher brain areas, including hippocampus and cerebral cortex, which are known to be critically involved in cognitive processes [10, 151]. Consistent with this observation, muscarinic agonist-induced activation of the MAPK pathway was virtually abolished in primary cortical cultures [152] or CA1 hippocampal pyramidal neurons from $M1R^{-/-}$ mice [153]. Similar results were obtained in [35 S]-GTP γ S binding assays [154] and in in vitro [152] and in vivo [155] studies examining muscarinic agonist-mediated stimulation of PI hydrolysis in hippocampal and cortical preparations. These findings clearly indicated that the M_1 receptor is the functionally predominant G_q -coupled mAChR subtype expressed in the forebrain.

Based on the high expression levels of the M_1 receptor in hippocampus and cerebral cortex, together with the outcome of classical pharmacological studies using subtype-preferring mAChR antagonists, it has been speculated that M_1 receptors may play a role in mediating higher cognitive processes such as learning and memory [156, 157]. To test this hypothesis, Miyakawa et al. [158] subjected $M1R^{-/-}$ mice to several hippocampus-dependent learning and memory tasks. $M1R^{-/-}$ mice performed equally well as their WT littermates in the Morris water maze, a test which is frequently used to assess spatial reference memory in rodents. Moreover, $M1R^{-/-}$ mice displayed normal freezing levels during context testing carried out 24 h after fear conditioning and did not show any significant cognitive deficits in the eight-arm radial maze test during training with 30–120 s delay time between individual trials [158]. On the other hand, $M1R^{-/-}$ mice showed performance deficits in the eight-arm radial maze test during trials without delay and during auditory-cued and context testing carried out 48 h and four weeks, respectively, after fear conditioning [158]. However, the extent of these behavioral deficits showed a very good correlation with the degree of hyperactivity displayed by the tested $M1R^{-/-}$ mice (see Section 5.14.1.3), suggesting that the hyperactivity phenotype exhibited by the $M1R^{-/-}$ mice makes a major contribution to the observed performance deficits. Interestingly, the behavioral pattern displayed by the $M1R^{-/-}$ mice is somewhat reminiscent of human attention-deficit-hyperactivity disorder in which hyperactivity is often accompanied by cognitive deficits [159].

Anagnostaras et al. [160] recently reported that $M1R^{-/-}$ mice displayed both enhancements and impairments of distinct cognitive functions. These authors found that $M1R^{-/-}$ mice exhibited a mild reduction in hippocampal long-term potentiation (LTP) in response to theta burst stimulation (Schaffer-CA1 synapse). $M1R^{-/-}$ mice showed normal or enhanced memory for tasks that involved matching-to-sample problems (contextual fear conditioning and Morris water maze). In contrast, $M1R^{-/-}$ mice displayed significant impairments in non-matching-to-sample working memory and consolidation (win-shift radial arm and social discrimination learning). Based on these observations, Anagnostaras et al. [160] concluded that M_1 receptors are not essential for memory formation or initial stability of memory in the hippocampus but are most likely involved in processes requiring interactions between cerebral cortex and hippocampus.

In a recent study, Seeger et al. [161] examined the potential role of M_2 receptors in learning and memory and hippocampal synaptic plasticity. Interestingly, $M2R^{-/-}$ mice showed significant deficits in behavioral flexibility and working memory in the

Barnes circular maze and the T-maze delayed alternation test, respectively. Moreover, the absence of M_2 receptors led to profound changes in neuronal plasticity studied at the Schaffer-CA1 synapse using hippocampal slices. Strikingly, short-term potentiation (STP) was abolished and LTP was drastically reduced following high-frequency stimulation of $M2R^{-/-}$ hippocampi. Incubation of $M2R^{-/-}$ hippocampal slices with bicuculline, a γ -aminobutyric acid ($GABA_A$) receptor antagonist, restored STP and significantly increased LTP. This observation, together with results obtained in whole-cell recordings from CA1 pyramidal cells, suggested that the deficits in synaptic plasticity observed with $M2R^{-/-}$ hippocampi are most likely caused by enhanced GABAergic inhibition. Seeger et al. [161] also demonstrated that the persistent enhancement of excitatory synaptic transmission in CA1 pyramidal cells induced by the transient application of low concentrations of a muscarinic agonist (referred to as LTP_m [162]) was totally abolished in $M2R^{-/-}$ mice.

In vivo microdialysis studies showed that $M2R^{-/-}$ mice displayed significant changes in pharmacologically and physiologically evoked ACh release in the hippocampus, consistent with the role of presynaptic M_2 autoreceptors in mediating inhibition of hippocampal ACh release [163]. These neurochemical changes were accompanied by performance deficits in a passive avoidance test [163], suggesting that improper regulation of synaptic ACh release may also contribute to the cognitive deficits caused by the lack of M_2 receptors.

Since impaired central muscarinic signaling is associated with Alzheimer's disease and normal aging processes [164], these findings should be of considerable therapeutic relevance.

5.14.1.2 Epileptic Seizures. Hamilton et al. [87] examined the ability of pilocarpine, a non-subtype-selective muscarinic agonist, to induce epileptic seizures in WT and $M1R^{-/-}$ mice. Whereas pilocarpine consistently elicited seizures in WT mice, it was completely devoid of seizure activity in $M1R^{-/-}$ mice. The lack of M_2 – M_5 receptors did not interfere with pilocarpine-induced seizure responses [155]. These results raise the possibility that increased signaling through central M_1 receptors may play a role in the pathophysiology of at least certain forms of epileptic seizures.

5.14.1.3 Locomotor Activity. Interestingly, $M1R^{-/-}$ mice showed a pronounced increase in locomotor activity that was consistently observed in all tests that included locomotor activity measurements [158]. Gerber et al. [165] reported that the hyperactivity phenotype of the $M1R^{-/-}$ mice was associated with a significant increase (approximately two-fold) in extracellular dopamine concentrations in the striatum, most probably due to an increase in dopamine release. It remains unclear at present whether this activity is mediated by intra- or extrastriatal M_1 receptors. Independent of the precise mechanism by which the lack of M_1 receptors leads to an increase in locomotor activity and striatal dopamine release, these findings suggest that centrally active, selective M_1 mAChR antagonists are potentially useful in the treatment of Parkinson's disease, a brain disorder characterized by drastically reduced striatal dopamine levels. Since schizophrenia is associated with increased dopaminergic transmission in various forebrain areas, improper signaling through M_1 receptors may contribute to the pathophysiology of certain forms of schizophrenia [165].

M4R^{-/-} mice also displayed a small but statistically significant increase in basal locomotor activity [166]. Interestingly, the locomotor stimulation observed after administration of a centrally active D1 dopamine receptor agonist was greatly enhanced in M4R^{-/-} mice [166]. In the striatum, M₄ mAChRs are coexpressed with D1 dopamine receptors by striatal projection neurons which give rise to the so-called direct striatonigral pathway activation which facilitates locomotion [167]. The findings by Gomez et al. [166] therefore suggest that striatal M₄ receptors exert an inhibitory effect on D1 receptor-stimulated locomotor activity. Since functional interactions between striatal cholinergic and dopaminergic pathways play a key role in extrapyramidal motor control [167], these observations are of potential relevance for the treatment of Parkinson's disease and related movement disorders.

5.14.1.4 Analgesia. Administration of centrally active muscarinic agonists induces robust analgesic effects which are dependent on both spinal and supraspinal mechanisms [168–170]. Using the tail-flick and hot-plate analgesia tests, Gomez et al. [131, 166] examined whether muscarinic agonist-induced analgesic responses were altered in M2R^{-/-} and M4R^{-/-} mice. These studies showed that the analgesic potency of oxotremorine administered systemically (s.c.) was markedly reduced (but not abolished) in M2R^{-/-} mice [131] but remained nearly unchanged in M4R^{-/-} mice [166]. Similar responses were observed after intrathecal (i.t.) or intracerebroventricular (i.c.v.) administration of oxotremorine [171]. Strikingly, oxotremorine was virtually devoid of analgesic activity in M2R^{-/-}/M4R^{-/-} double-KO mice [171], independent of the route of application (s.c., i.t., or i.c.v.). These findings indicate that both M₂ receptors (which functionally predominate) and M₄ receptors are involved in mediating the analgesic effects of muscarinic agonists at the spinal and supraspinal levels. Most likely, the antinociceptive effects mediated by M₄ receptors remained undetected in the M4R^{-/-} mice [166] due to the presence of the functionally predominant M₂ receptor. Radioligand binding studies with spinal cord tissues from WT and mAChR mutant mice suggested that ~90% of all mAChRs in the spinal cord represent M₂ receptors [171]. The precise mechanisms by which spinal and supraspinal M₂ and M₄ receptors mediate their analgesic effects remain to be elucidated.

Duttaroy et al. [171] also reported that two novel muscarinic agonists chemically derived from epibatidine, CMI-936 and CMI-1145 [172], showed significantly higher affinity (~6–16-fold) for M₄ than for M₂ receptors and displayed reduced analgesic activity in both M2R^{-/-} and M4R^{-/-} mice, independent of the route of application. Consistent with the outcome of the analgesia experiments, [³⁵S]GTPγS binding studies using tissues from different mAChR mutant mice also demonstrated the existence of a small but functionally significant M₄ receptor population in the mouse spinal cord [173]. Since M₄ receptors, unlike M₂ receptors, do not seem to mediate important peripheral functions, the potential use of selective M₄ receptor agonists as novel analgesic agents is unlikely to be associated with any significant peripheral side effects. It should also be noted that the potential use of muscarinic agonists as analgesic drugs is probably less likely to lead to tolerance and addiction associated with the use of classical opioid analgesics [170, 174].

5.14.1.5 Muscarinic Agonist-induced Tremor and Hypothermia. Systemic administration of oxotremorine or other centrally active muscarinic agonists causes

whole-body tremor and pronounced hypothermic effects. Strikingly, Gomeza et al. [131] found that oxotremorine-mediated tremor was totally abolished in $M2R^{-/-}$ mice. Pharmacological antagonism of oxotremorine-induced tremor activity has often been used as a model system for identifying new anti-Parkinson drugs. The findings by Gomeza et al. [131] therefore suggest that drugs originating from this screening procedure were selected based on their ability to block central M_2 receptors.

Moreover, oxotremorine-induced hypothermic responses were significantly reduced but not abolished in $M2R^{-/-}$ mice [131], suggesting that both M_2 and non- M_2 mAChRs may play a role in cholinergic regulation of body temperature.

5.14.1.6 Role of M_3 mAChRs in Stimulating Food Intake. Yamada et al. [175] reported that $M3R^{-/-}$ mice showed a pronounced reduction in body weight (by $\sim 25\%$) that was associated with a significant decrease in the mass of peripheral fat pads and greatly reduced serum leptin and insulin levels. Food intake studies showed that $M3R^{-/-}$ mice consumed considerable less food than their WT littermates [175]. Analysis of the expression levels of various hypothalamic neuropeptides known to play key roles in the regulation of appetite showed that $M3R^{-/-}$ mice displayed greatly reduced expression levels of melanin-concentrating hormone (MCH). MCH is virtually exclusively expressed in so-called second-order neurons of the lateral hypothalamus and is normally increased in fasted mice or under conditions of leptin deficiency [176]. Interestingly, MCH-containing neurons express M_3 receptors [175], and muscarinic stimulation increases hypothalamic MCH expression [177]. Yamada et al. [175] also showed that i.c.v. administration of the appetite-stimulating, agouti-related peptide, which is known to stimulate the activity of MCH-containing hypothalamic neurons [176], failed to stimulate food intake in $M3R^{-/-}$ mice. Taken together, these observations suggest that activation of hypothalamic M_3 receptors expressed by MCH neurons plays a role in the stimulation of food intake. Pharmacological manipulation of this hypothalamic cholinergic pathway may therefore represent a novel strategy for the control of food intake. It should be noted, however, that M_3 receptors are also widely expressed in peripheral organs including smooth muscle and glandular tissues. Thus, additional studies are needed to assess the relative contribution of central versus peripheral deficits to the hypophagia phenotype displayed by the $M3R^{-/-}$ mice.

5.14.1.7 Central Muscarinic Autoreceptors. ACh, like many other neurotransmitters, can inhibit its own release via stimulation of so-called inhibitory autoreceptors present on cholinergic nerve endings [178]. Using superfused hippocampal, cortical, and striatal slices that had been preincubated with [3 H]choline to label cellular ACh pools, Zhang et al. [179] demonstrated that autoinhibition of ACh release is mediated predominantly by M_2 receptors in the mouse hippocampus and cerebral cortex, but primarily by M_4 receptors in the mouse striatum. Since the proper regulation of ACh release in these tissues is thought to be critically involved in various fundamental functions of the CNS, including cognition and locomotor control, these findings should be of high clinical relevance.

5.14.1.8 Modulation of Striatal Dopamine Release. Activation of striatal mAChRs is known to stimulate dopamine release in the striatum [180, 181]. Studies with superfused striatal slices demonstrated that oxotremorine-mediated potentiation of

potassium-stimulated [^3H]dopamine release was abolished in $\text{M4R}^{-/-}$ mice, significantly increased in $\text{M3R}^{-/-}$ mice, and significantly reduced (but not abolished) in $\text{M5R}^{-/-}$ mice [182, 183]. Additional studies suggested that the dopamine release-stimulating M_4 receptors are probably located on neuronal cell bodies but that the release-facilitating M_5 and the release-inhibiting M_3 receptors are most likely located on nerve terminals [183]. Pharmacological blockade of striatal GABA_A receptors suggested that M_3 and M_4 receptors mediate their dopamine release-modulatory effects via facilitation or inhibition, respectively, of striatal GABA release [183]. Since striatal dopaminergic signaling is severely impaired in Parkinson's disease, these findings should be of considerable therapeutic interest.

5.14.1.9 Suppression of Inhibitory Hippocampal Synapses. Electrophysiological studies carried out with hippocampal neurons prepared from different mAChR single- and double-KO mice recently identified two distinct mechanisms by which muscarinic activation can suppress inhibitory synaptic transmission in the hippocampus [184, 185]. Activation of M_2 mAChRs located on presynaptic terminals was found to be required for the suppression of GABA release in a cannabinoid receptor type 1 (CB1)–independent fashion [185]. Additional evidence suggested that activation of a mixture of postsynaptic M_1 and M_3 mAChRs facilitates the depolarization-induced release of endocannabinoids which are predicted to suppress GABA release via activation of presynaptic CB1 receptors [184, 185].

5.14.1.10 M_4 Receptor-mediated Regulation of Prepulse Inhibition and Haloperidol-Induced Catalepsy. Phenotypical analysis of $\text{M4R}^{-/-}$ mice suggested that central M_4 receptors play a role in modulating prepulse inhibition (PPI) of the startle reflex, a measure of attention [186]. Specifically, Felder et al. [186] demonstrated that $\text{M4R}^{-/-}$ mice displayed a significant increase in sensitivity to the PPI-disrupting effect of the psychomimetic phencyclidine, a noncompetitive *N*-methyl-d-aspartate (NMDA) receptor antagonist. Since phencyclidine-mediated disruption of PPI is frequently used as an animal model of psychosis, central M_4 receptors may represent a novel drug target for the treatment of schizophrenia and related neurological disorders.

In a recent study, Karasawa et al. [187] examined the ability of the muscarinic antagonist scopolamine to suppress the cataleptic responses induced by the D2-type dopamine receptor antagonist haloperidol in WT and $\text{M4R}^{-/-}$ mice. While scopolamine abolished haloperidol-induced catalepsy in WT mice, it had little effect on haloperidol-mediated cataleptic responses in $\text{M4R}^{-/-}$ mice [187]. Since haloperidol-induced catalepsy is often used as an animal model to mimic the extrapyramidal motor side effects caused by antipsychotic drugs, these findings suggest that selective blockade of M_4 receptors may be beneficial in treating the extrapyramidal symptoms in Parkinson's disease and related movement disorders.

5.14.1.11 Role of M_4 and M_5 Receptors in Modulating Dopamine Efflux in Nucleus Accumbens. Forster et al. [188] demonstrated that electrical stimulation of the laterodorsal tegmental nucleus, a mesopontine nucleus that provides the major source of cholinergic input to the dopamine-containing neurons of the ventral tegmental area (VTA [189]), triggers a three-phasic pattern of changes in dopamine efflux in the nucleus accumbens (nAcc) of WT mice. Strikingly, the long-lasting

increase in dopamine levels in the nAcc (phase III) was selectively abolished in M5R^{-/-} mice [188]. This neurochemical deficit is most likely caused by the lack of excitatory M₅ receptors expressed by the dopamine-containing VTA neurons which predominantly express the M₅ mAChR subtype [19].

In another in vivo microdialysis study, Tzavara et al. [190] showed that basal dopamine levels were significantly increased in the nAcc of M4R^{-/-} mice. Enhanced dopamine efflux in the nAcc of M4R^{-/-} mice was also observed after administration of the psychostimulants *d*-amphetamine and phencyclidine [190]. These neurochemical alterations were associated with significantly increased basal ACh levels in the midbrain of M4R^{-/-} mice. Moreover, in the absence of M₄ receptors, the enhancement of midbrain ACh efflux in response to scopolamine was markedly reduced [190]. These findings are consistent with the concept that the absence of inhibitory midbrain M₄ autoreceptors is responsible for the enhanced dopamine efflux in the nAcc of M4R^{-/-} mice, probably due to increased excitatory cholinergic flow to midbrain dopaminergic neurons. Centrally active M₄ receptor agonists may therefore become clinically useful in the treatment of CNS disorders associated with hyperdopaminergia.

5.14.1.12 Role of M₅ Receptors in Modulating Rewarding Effects of Drugs of Abuse. Consistent with the observation that activation of mesolimbic M₅ receptors facilitates dopamine release in the nAcc [188], behavioral studies demonstrated that M5R^{-/-} mice showed reduced sensitivity to the rewarding effects of morphine and cocaine [191, 192]. In addition, the severity of morphine and cocaine withdrawal symptoms was shown to be reduced in M5R^{-/-} mice [191, 192]. These behavioral deficits were associated with distinct biochemical changes in the nAcc, including a reduction in morphine-stimulated dopamine efflux and Fos-B expression [191]. Taken together, these findings suggest that centrally active M₅ receptor antagonists may become therapeutically useful for the treatment of drug addiction.

5.14.2 Functions Mediated by Peripheral mAChRs

5.14.2.1 Smooth Muscle. Studies with isolated smooth muscle tissues from M3R^{-/-} mice demonstrated that M₃ mAChRs play a predominant role in mediating muscarinic agonist (ACh)-induced smooth muscle contractions [40, 148], consistent with the outcome of a large number of pharmacological studies [193]. For example, in vitro studies showed that smooth muscle tissues (urinary bladder, ileum, stomach fundus, trachea, and gallbladder preparations) derived from M3R^{-/-} mice exhibited significantly reduced maximum contractile responses (*E*_{max}) following addition of the cholinergic agonist carbachol [194–196]. However, the extent of reduction in *E*_{max} values differed significantly among tissues, ranging from only ~40% in tracheal smooth muscle [195] to >90% in urinary bladder [194].

In vivo studies showed that M3R^{-/-} mice had enlarged pupils [194], indicating that the tone of the pupillary sphincter muscle is maintained by tonic activation of M₃ receptors. However, M3R^{-/-} mice retained a weak light reflex, and atropine was able to further increase pupil size in M3R^{-/-} mice [194], suggesting that non-M₃ mAChRs also contribute to modulating ocular smooth muscle contractility. Male but not female M3R^{-/-} mice also exhibited severely distended urinary bladders [194]. However, in vivo studies demonstrated that gastrointestinal function including

food transit time is normal in $M3R^{-/-}$ mice [175, 194], suggesting that the presence of M_3 receptors is not essential for gastrointestinal motor activity in vivo.

Stengel et al. [197] showed that carbachol was about twofold less potent in contracting isolated smooth muscle preparations from stomach fundus, urinary bladder, and trachea from $M2R^{-/-}$ mice, as compared to the corresponding WT preparations. Strikingly, Matsui et al. [198] reported that carbachol-mediated contractions were almost completely abolished in ileal and urinary bladder preparations from mice deficient in both M_2 and M_3 mAChRs ($M2R^{-/-}/M3R^{-/-}$ mice). This observation strongly suggests that muscarinic agonist-mediated smooth muscle contractions are mediated by a mixture of M_3 receptors (which clearly predominate functionally) and M_2 receptors.

In a related study, Struckmann et al. [199] demonstrated that cholinergic constriction of murine peripheral airways is also mediated by a mixture of M_3 and M_2 receptors. This study also revealed the existence of pulmonary M_1 receptors whose activation counteracts cholinergic bronchoconstriction. Interestingly, a recent in vivo study showed that vagally or muscarinic agonist (methacholine)-induced bronchoconstrictor responses were abolished in $M3R^{-/-}$ mice [126]. Given the important role of muscarinic cholinergic mechanisms in pulmonary disease, these findings should be of considerable therapeutic relevance.

Matsui et al. [200] recently reported that the relaxant effects of forskolin were enhanced in different smooth muscle tissues from $M2R^{-/-}$ mice stimulated with the muscarinic agonist oxotremorine-M. This observation suggests that stimulation of smooth muscle M_2 receptors can counteract the relaxant effects of agents that increase cAMP levels, probably via M_2 receptor-induced activation of G proteins of the G_i family which mediate the inhibition of adenylyl cyclase. Taken together, these data indicate that M_2 receptors facilitate smooth muscle contractility through both direct and indirect mechanisms. In addition, a recent study using ileal segments prepared from $M2R^{-/-}$ and $M3R^{-/-}$ mice strongly suggested that muscarinic agonist-induced short-term heterologous desensitization of intestinal smooth muscle requires the simultaneous activation of both M_2 and M_3 mAChRs [201].

5.14.2.2 Salivary Secretion. Pharmacological evidence suggests that M_3 mAChRs play an important role in mAChR-mediated salivation [9]. Consistent with this concept, Matusi et al. [194] reported that injection of a single low dose of pilocarpine (1 mg/kg, s.c.) induced salivation in WT mice but failed to do so in $M3R^{-/-}$ mice. However, subsequent studies showed that administration of higher doses of pilocarpine led to pronounced salivation responses in $M3R^{-/-}$ mice [149, 175, 202]. Gautam et al. [149] recently showed that pilocarpine-induced salivation responses were abolished in $M1R^{-/-}/M3R^{-/-}$ double-KO mice. Similar results were obtained by Nakamura et al. [202] studying an independently generated $M1R^{-/-}/M3R^{-/-}$ mutant mouse line. Taken together, these studies support the concept that cholinergic stimulation of salivary flow is mediated by a mixture of M_1 and M_3 receptors and that other mAChRs do not contribute to this activity to a significant extent.

5.14.2.3 Insulin and Glucagon Secretion from Pancreatic Islets. Pancreatic mAChRs play an important role in stimulating insulin secretion from pancreatic β cells [203]. Studies with isolated pancreatic islets prepared from WT and $M3R^{-/-}$ mice recently

showed that muscarinic agonist-mediated enhancement of glucose-dependent insulin release was abolished in $M3R^{-/-}$ mice [204, 205]. Duttaroy et al. [204] also demonstrated that muscarinic agonist-induced glucagon release (glucagon is expressed by pancreatic α cells) was also greatly diminished in islets from $M3R^{-/-}$ mice. These findings indicate that strategies aimed at enhancing the activity of β -cell M_3 receptors or M_3 receptor-regulated downstream signaling components may represent a useful novel approach to promote insulin release in type 2 diabetes.

5.14.2.4 Amylase Secretion from Exocrine Pancreas. Activation of pancreatic mAChRs also plays an important role in stimulating the secretion of digestive enzymes from the exocrine pancreas. Gautam et al. [150] recently showed that carbachol-induced stimulation of amylase secretion was significantly impaired in pancreatic acinar preparations from both $M1R^{-/-}$ and $M3R^{-/-}$ receptor single-KO mice and completely abolished in acinar preparations from $M1R^{-/-}/M3R^{-/-}$ double-KO mice. However, another pancreatic secretagogue, bombesin, retained its ability to fully stimulate amylase secretion in acinar preparations from $M1R^{-/-}/M3R^{-/-}$ double-KO mice. Carbachol was significantly less potent in stimulating amylase release in acinar preparations from $M3R^{-/-}$ than from $M1R^{-/-}$ mice [150], suggesting that the M_3 receptor plays a predominant role in mediating pancreatic exocrine secretion. These studies support the concept that cholinergic stimulation of pancreatic amylase secretion is mediated by a mixture of M_1 and M_3 mAChRs and that other mAChR subtypes do not make a significant contribution to this activity.

5.14.2.5 Gastric Acid Secretion. mAChRs are known to play an important role in the regulation of gastric acid secretion. Studies with $M3R^{-/-}$ mice showed that M_3 receptor activity is essential for the basal secretion of gastric acid and the trophic response of the oxyntic mucosa to gastrin [206]. Moreover, gastric acid output in response to administration of carbachol, histamine, or gastrin was significantly reduced in $M3R^{-/-}$ mice [206]. On the other hand, the lack of M_1 receptors had no significant effect on the magnitude of these responses [207]. Surprisingly, $M5R^{-/-}$ mice showed a reduction in both carbachol-mediated gastric acid secretion and carbachol-induced histamine release [207]. Since M_5 receptor mRNA was found in the stomach but not in the fundic or antral mucosa, Aihara et al. [207] concluded that gastric acid secretion is mediated by activation of M_3 receptors located on gastric parietal cells and of M_5 receptors present in the gastric submucosal plexus.

5.14.2.6 Cardiovascular System

5.14.2.6.1 Heart. ACh released from cardiac vagal nerve endings interacts with mAChRs located in the sinoatrial node to trigger a reduction in heart rate [9]. In vitro studies showed that carbachol-mediated bradycardic responses were completely abolished in isolated spontaneously beating atria prepared from $M2R^{-/-}$ mice [197]. Moreover, a recent in vivo study demonstrated that vagally or muscarinic agonist (methacholine)-induced reductions in heart rate were also absent in $M2R^{-/-}$ mice [126]. Taken together, these studies provide unambiguous evidence that the negative chronotropic effects following vagal stimulation are mediated by M_2 mAChRs and that other mAChRs do not make a significant contribution to the regulation of heart rate.

5.14.2.6.2 Blood Vessels. ACh is able to dilate most vascular beds by interacting with mAChRs located on vascular endothelial cells [208, 209]. Interestingly, Yamada et al. [182] reported that ACh virtually lost the ability to dilate cerebral arteries (basilar artery) and arterioles (pial vessels) prepared from $M5R^{-/-}$ mice, suggesting that ACh relaxes cerebral arteries and arterioles via stimulation of endothelial M_5 receptors. Since neuronally released ACh plays a role in the regulation of cerebral vascular resistance and regional blood flow [210, 211], cerebrovascular M_5 mAChRs may therefore represent an attractive novel therapeutic target for the treatment of a variety of cerebrovascular disorders, including Alzheimer's disease and certain forms of cerebral ischemia.

On the other hand, analysis of mAChR mutant mice showed that the ACh-induced relaxation of coronary arteries and the aorta is mediated predominantly by the M_3 receptor subtype [212, 213].

5.14.2.6.3 Cardiovascular Effects of McN-A-343. Systemic administration of McN-A-343, a muscarinic agonist that can activate M_1 mAChRs located on postsynaptic sympathetic ganglion neurons with high efficacy [214], caused stimulatory cardiovascular effects in WT mice but failed to do so in $M1R^{-/-}$ mice [215]. This observation convincingly demonstrates that McN-A-343 mediates its stimulatory cardiovascular effects via activation of M_1 receptors.

5.14.2.7 Peripheral Muscarinic Autoreceptors and Heteroreceptors. Recently, mAChR mutant mice have also served as tools to identify the mAChR subtypes involved in mediating autoinhibition of ACh release in various peripheral tissues. Zhou et al. [216] demonstrated that the mAChRs mediating autoinhibition of ACh release in mouse heart atria represent a mixture of M_4 and non- M_4 (probably M_2) receptors. In the mouse urinary bladder, autoinhibition of ACh release was found to be mediated predominantly by M_4 receptors [216]. Takeuchi et al. [217] showed that autoinhibition of ACh release from the mouse longitudinal muscle/myenteric plexus preparation is mediated by a mixture of M_2 and M_4 mAChRs.

A recent study examining neurotransmitter release from phrenic diaphragm preparations of WT and $M2R^{-/-}$ mice demonstrated that the inhibitory muscarinic autoreceptors present on peripheral cholinergic motor nerves represent M_2 receptors [218]. These authors also provided data suggesting that presynaptic M_2 receptors play a role in controlling the time course of ACh release from the terminals of cholinergic motor neurons [218].

Activation of mAChRs located on peripheral sympathetic nerve terminals (so-called muscarinic heteroreceptors) results in the inhibition of norepinephrine release [219]. To study the molecular identity of these muscarinic heteroreceptors, Trendelenburg et al. [220] examined various peripheral preparations from WT and mAChR mutant mice. Specifically, these investigators determined electrically evoked [3 H]norepinephrine release using cardiac (atrial), urinary bladder, and vas deferens tissues from WT, $M2R^{-/-}$, and $M4R^{-/-}$ mice. This analysis showed that the release-inhibitory muscarinic heteroreceptors represent mixtures of M_2 and non- M_2 receptors in all three tissues studied. Whereas the non- M_2 heteroreceptors present in the vas deferens are likely to represent primarily M_4 receptors, the identity of the non- M_2 heteroreceptors present in heart atria and urinary bladder remains uncertain [220].

5.14.2.8 Role of M_1 mAChRs in Development of Cytolytic T Cells. Several lines of evidence suggest that ACh plays an immunoregulatory role by modulating the function of T lymphocytes (see [221] and references therein). Zimring et al. [221] recently showed that activation of mAChRs can trigger the generation of $CD8^+$ cytolytic T lymphocytes. Interestingly, $CD8^+$ T cells prepared from $M1R^{-/-}$ mice showed a defect in the ability to differentiate into cytolytic T lymphocytes, whereas $CD8^+$ T cells from other mAChR mutant mice responded normally [221]. These findings support the novel concept that M_1 mAChRs play a role in the development of cytolytic T cells.

5.14.2.9 Functions of mAChRs in Skin. Multiple mAChR subtypes are known to be expressed by different cell types of the skin [222, 223]. Studies with $M3R^{-/-}$ mice including the use of M_3 receptor antisense oligonucleotides suggested that M_3 mAChRs play a role in mediating skin keratinocyte adhesion, most probably by modulating cadherin and catenin levels and activities [224]. Moreover, in vitro and in vivo studies with mAChR mutant mice demonstrated that keratinocyte migration and wound reepithelialization are facilitated by activation of M_4 and inhibited by activation of M_3 mAChRs [223]. These findings may lead to the development of novel therapeutic strategies useful for promoting wound healing.

Activation of mAChRs present on peripheral nociceptors of the skin can suppress the transmission of pain impulses [225, 226]. Strikingly, muscarine-induced peripheral antinociception was abolished in $M2R^{-/-}$ but not significantly affected in $M4R^{-/-}$ mice, as shown in electrophysiological and neurochemical studies using skin and skin-saphenous nerve preparations [227]. It is possible that activation of these peripheral M_2 receptors contributes to the analgesic responses observed after systemic administration of muscarinic agonists (see Section 5.14.1.4).

5.15 CONCLUSIONS

Studies with M_1 – M_5 mAChR single-KO and various mAChR double-KO strains have led to a wealth of novel information about the physiological and pathophysiological roles of the individual mAChR subtypes. The development of a new generation of mAChR mutant mice in which specific receptor subtypes can be inactivated in a tissue-specific and/or time-dependent fashion should provide even more powerful research tools. These studies should pave the way towards the development of novel strategies for the treatment of a variety of important pathophysiological conditions including pain, obesity, diabetes, and various disorders of the CNS.

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6

NOREPINEPHRINE/EPINEPHRINE

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6.1	Historical Perspective	194
6.2	Physiology	197
6.3	Neurochemistry	198
6.4	Norepinephrine Transporter	199
6.4.1	General Characteristics of NET	199
6.4.2	Genetic Variations in Norepinephrine Transporter Gene	200
6.5	α_1 -Adrenergic Receptors	201
6.5.1	General Characteristics and Regulation of α_1 -Adrenergic Receptors	201
6.5.2	Signal Transduction Pathways for α_1 -Adrenergic Receptors	203
6.5.3	Physiological Roles for α_1 -Adrenergic Receptors	203
6.6	α_2 -Adrenergic Receptors	204
6.6.1	General Characteristics and Regulation of α_2 -Adrenergic Receptors	204
6.6.2	Signal Transduction Pathways for α_2 -Adrenergic Receptors	206
6.6.3	Physiological Roles for α_2 -Adrenergic Receptors	207
6.7	β -Adrenergic Receptors	209
6.7.1	General Characteristics and Regulation of β -Adrenergic Receptors	209
6.7.2	Signal Transduction Pathways for β -Adrenergic Receptors	210
6.7.3	Physiological Roles for β -Adrenergic Receptors	211
	References	213

Norepinephrine (noradrenaline) is a neurotransmitter in both the peripheral and central nervous systems. Epinephrine (adrenaline) is a hormone released from the adrenal gland. Norepinephrine and epinephrine are catecholamines, because they have both the catechol moiety (two hydroxyl groups on a benzene ring) and an amine (NH_2) (see Fig. 6.1). Both of these catecholamine messengers play important roles in the regulation of diverse physiological systems by acting through adrenergic receptors. Stimulation of adrenergic receptors by catecholamines released in response to activation of the sympathetic autonomic nervous system results in a variety of effects such as increased heart rate, regulation of vascular tone, and bronchodilation. In the central nervous system (CNS), adrenergic receptors are involved in many functions, including memory, learning, alertness, and the response to stress.

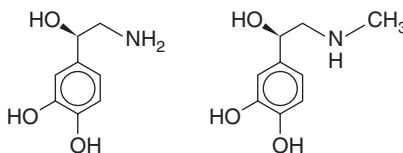


Figure 6.1 Molecular structures of norepinephrine and epinephrine.

6.1 HISTORICAL PERSPECTIVE

Epinephrine was first isolated from the adrenal gland in the late 1890s (later shown to be as a benzoyl derivative) by John Jacob Abel, who is the father of American pharmacology and is generally credited with isolation of the first hormone [1]. A few years later, epinephrine was isolated as the free base (later shown to be contaminated with norepinephrine) by Jokichi Takamine. Norepinephrine was synthesized in 1904 by Friedrich Stolz, who also made the first observations on its biological properties; it was as active as epinephrine in raising the blood pressure of animals but less toxic [2]. Little interest was shown in norepinephrine at that time, but two years later it was demonstrated that it possessed remarkable pressor actions and had a lower toxicity compared with epinephrine.

Starting in the 1920s Cannon attempted to identify the chemical transmitter of the sympathetic nervous system (which he called sympathin) and mistakenly concluded in 1933 that there were two sympathins, sympathin E (excitatory) and sympathin I (inhibitory) [3]. This was due in part to the fact that he was using a natural preparation, adrenaline, which at that time was a variable mixture of epinephrine and norepinephrine. It was not until the late 1940s that von Euler finally established that norepinephrine was the predominant postganglionic neurotransmitter of the sympathetic nervous system [4], for which he was awarded the Nobel Prize in 1970.

In the early 1960s Julius Axelrod, who was studying the metabolic fate of norepinephrine, injected the recently available [^3H]norepinephrine into rats and cats. He noted that the radioactivity concentrated in tissues enriched in sympathetic nerve endings but that lesions of the sympathetic nerves abolished the accumulation. Based on these observations, Axelrod then showed that norepinephrine is inactivated by a “reuptake” system that involves the transmitter being pumped back into the nerve ending that had released it [5]. Neurotransmitter inactivation had been thought to be primarily enzymatic (based on acetylcholine), but it soon became evident that reuptake was the main route of inactivation for most neurotransmitters. Axelrod also discovered that drugs such as cocaine and imipramine inhibited the reuptake of norepinephrine [6]. For his elucidation of the reuptake process Axelrod was awarded the Nobel Prize, also in 1970.

Adrenergic receptors were originally divided into two major types, α and β , based on their pharmacological characteristics (i.e., rank-order potency of agonists) [7]. Subsequently, it became apparent that there were actually three major types (Fig. 6.2), α_1 , α_2 and β [8], each of which has three subtypes. Adrenergic receptors belong to the superfamily of seven-transmembrane receptors, which consist of a

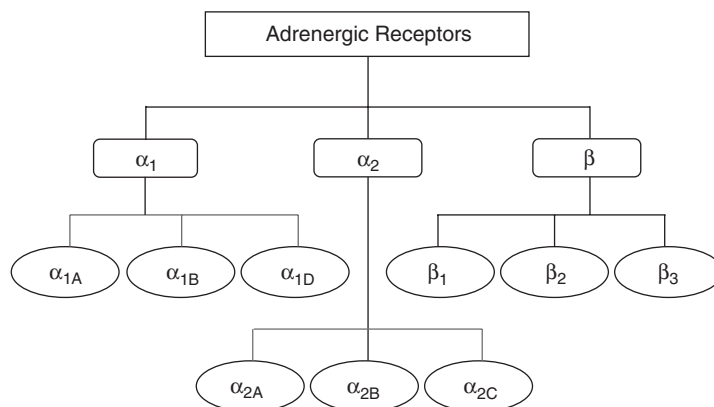


Figure 6.2 Classification scheme for adrenergic receptors. (Reprinted from [7a].) (See color insert.)

single polypeptide chain with seven hydrophobic regions that are thought to form α helical structures and span or transverse the membrane. According to the official nomenclature of the International Union of Basic and Clinical Pharmacology (IUPHAR) as determined by the IUPHAR Committee on Receptor Nomenclature and Drug Classification, the adrenergic receptors are classified as 2.1 ADR [9]. The 2.1 means that they are in the rhodopsin subclass of the seven-transmembrane receptor superfamily (or main receptor structure class). The ADR is the receptor family code for norepinephrine/epinephrine (noradrenaline/adrenaline).

Because the mechanism of action of adrenergic receptors includes the activation of guanine nucleotide regulatory binding proteins (G proteins), they are also called G-protein-coupled receptors (GPCRs). The binding of norepinephrine (or another agonist) to an adrenergic receptor induces (or stabilizes) a conformational change which allows the receptor to interact with and activate a G protein. The activated receptor facilitates the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), leading to the dissociation of the α and $\beta\gamma$ subunits of the G protein, which in turn stimulate or inhibit the activity of various effectors (Fig. 6.3). Importantly, each of the three types of adrenergic receptors couples to a distinct class of G proteins: α_1 to G_q ; α_2 to $G_{i/o}$, and β to G_s . In addition to G proteins, adrenergic receptors interact with other signaling proteins and pathways such as those involving tyrosine kinases.

The β -adrenergic receptors were subdivided into β_1 (2.1.ADR.7.B1) and β_2 (2.1.ADR.8.B2) subtypes [10]. The β_1 -adrenergic receptor, the dominant receptor in heart and adipose tissue, is equally sensitive to epinephrine and norepinephrine, whereas the β_2 -adrenergic receptor, responsible for relaxation of vascular, uterine, and airway smooth muscle, is less sensitive to norepinephrine as compared to epinephrine. Subsequently it became apparent that not all of the β -adrenergic receptor-mediated responses could be classified as either β_1 or β_2 , suggesting the existence of at least one additional β subtype [11]. The β_3 receptor (2.1.ADR.9.B3) is insensitive to the commonly used β -adrenergic receptor antagonists and was previously referred to as the “atypical” β -adrenergic receptor [12]. A β_4 receptor

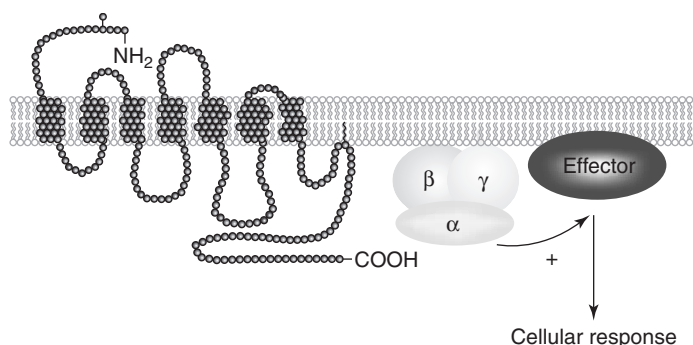


Figure 6.3 A GPCR and its signaling pathways. GPCRs can couple to a variety of heterotrimeric G proteins that are assembled from α (1 of 16), β (1 of 5) and γ (1 of 7) subunits. After G-protein coupling the G protein dissociates into α and $\beta\gamma$ subunits. The α and the $\beta\gamma$ subunits can modulate the activity of a variety of effector proteins—for example, adenylate cyclase, phospholipase A_2 , phospholipase C, or ion channels. (From [9a].) (See color insert.)

has been postulated; however, definitive evidence of its existence is lacking, and it is now thought to be a “state” of the β_1 adrenergic receptor [13].

The α -adrenergic receptors were first subdivided into postsynaptic (α_1) and presynaptic (α_2) subtypes [14]. After it was realized that not all α receptors with α_2 pharmacological characteristics were presynaptic (or prejunctional), the pharmacological definition was used [15]. Three genetic and four pharmacological α_2 adrenergic receptor subtypes have been defined. The evidence for α_2 receptor subtypes initially came from binding and functional studies in various tissues and cell lines [16]. The α_{2A} (2.1.ADR.4.A2A) and α_{2B} (2.1.ADR.5.A2B) subtypes were initially defined based on their differential affinity for adrenergic agents such as prazosin and oxymetazoline [17]. These subtypes were subsequently cloned from human, rat, mouse, and other species. A third subtype, α_{2C} (2.1.ADR.6.A2C), was identified originally in an opossum kidney cell line [18] and has also been cloned from several species. A fourth pharmacological subtype, the α_{2D} , was first identified in the rat and cow [19, 20]. This pharmacological subtype is a species ortholog of the human α_{2A} subtype and thus is not considered to be a separate genetic subtype.

Three genetic and four pharmacological α_1 -adrenergic receptor subtypes have also been defined [21]. The evidence for α_1 receptor subtypes initially came from binding and functional studies in various tissues and cell lines. The α_{1A} (2.1.ADR.1.A1A) and α_{1B} (2.1.ADR.2.A1B) subtypes were initially defined based on their differential affinity for adrenergic agents such as WB4101 and phentolamine [22] and their differential sensitivities to the site-directed alkylating agent chloroethylclonidine [23]. The α_{1B} subtype was subsequently cloned from the hamster and the α_{1A} was cloned from bovine brain, although it was originally called the α_{1C} -adrenergic receptor. A third subtype, α_{1D} (2.1.ADR.3.A1D) adrenergic receptor was subsequently cloned from the rat cerebral cortex, although this clone was originally called the α_{1a} -adrenergic receptor by some workers [21]. A fourth pharmacological subtype, the α_{1L} , has been identified in vascular tissues from several species [24] but may represent a conformational state of the α_{1A} receptor [25]. The current classification scheme includes the α_{1A} , the α_{1B} , and the α_{1D} , but there is no α_{1C} .

6.2 PHYSIOLOGY

Norepinephrine is a direct-acting sympathomimetic with pronounced effects on α_1 - and α_2 -adrenergic receptors and less marked effects on β -adrenergic receptors. Epinephrine, by contrast, has important β -adrenergic effects, particularly at lower concentrations. The effects of norepinephrine and epinephrine are primarily but not entirely excitatory, as reflected in the physical and mental arousal such as that seen in the fight-or-flight response. Norepinephrine is stored in granules in adrenergic nerve axons and released upon depolarization of the nerve. Epinephrine together with norepinephrine is present in and released from the adrenal medulla. The ratio of epinephrine to norepinephrine present in the adrenal gland is species dependent, with the ratio in the human being approximately 80 : 20.

A major effect of exogenously administered norepinephrine is increased systolic and diastolic blood pressure, which is accompanied by reflex slowing of the heart rate. This is a result of its α_1 stimulant effects which cause vasoconstriction, with reduced blood flow in the kidneys, liver, skin, and usually skeletal muscle. Norepinephrine causes the pregnant uterus to contract and high doses release glucose from the liver and have other hormonal effects similar to those of epinephrine. It produces little stimulation of the CNS. The β -adrenergic effects of epinephrine and to a lesser extent norepinephrine include positive inotropic and chronotropic actions on the heart and bronchodilatation in the lungs.

Norepinephrine is one of the “stress hormones” and endogenously affects parts of the human brain where attention and impulsivity are controlled. Physiological changes activated by a stressful event are mediated in part by activation of a nucleus in the brain stem called the locus ceruleus, which contains the cell bodies of nearly all of the noradrenergic neurons in the brain. These neurons project bilaterally from the locus ceruleus to most areas of the CNS, including the cortex, limbic system, and spinal cord (Fig. 6.4).

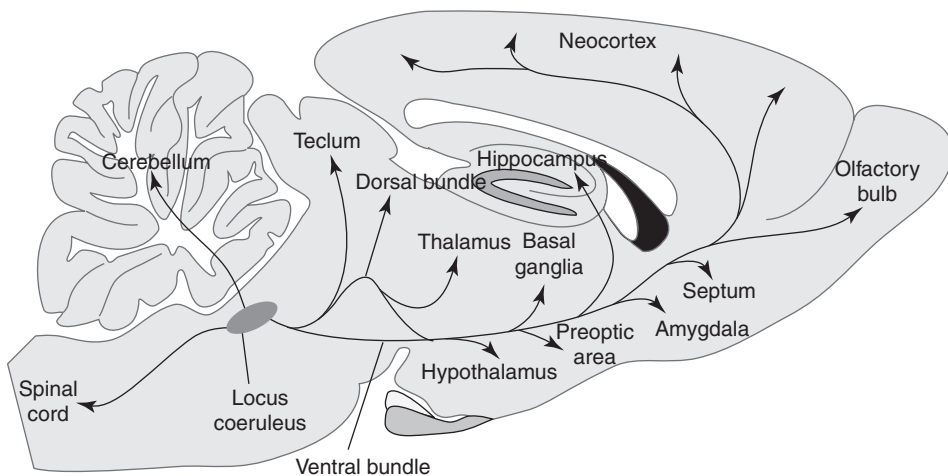


Figure 6.4 Midsagittal section of a rat brain showing locations of most important groups of noradrenergic neurons and distribution of their axons and terminal buttons. (From [25a].) (See color insert.)

A decrease in noradrenergic function due to a loss of locus ceruleus neurons is seen in Parkinson's and Alzheimer's diseases [26, 27]. A marked loss of noradrenergic fibers in the hippocampal formation of individuals with Alzheimer's disease correlates with the loss of cell bodies in the locus ceruleus [28].

6.3 NEUROCHEMISTRY

Norepinephrine and epinephrine are synthesized starting with the amino acid tyrosine, which is obtained from the diet and can also be synthesized from phenylalanine. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (EC 1.14.16.2). This is the rate-limiting and first committed step in the synthesis of norepinephrine and epinephrine and [29] is tightly regulated at multiple levels [29]. Disruption of the tyrosine hydroxylase gene (knockout mice) results in midgestational lethality [30]. DOPA, in turn, is decarboxylated by L-aromatic amino acid decarboxylase to form dopamine. As the name implies, this is a relatively nonselective enzyme and decarboxylates many substrates. These first two steps in the synthesis of norepinephrine and epinephrine take place in the cytoplasm of the neuron. Dopamine is transported into storage vesicles in the nerve terminals via amine-specific transporters and is hydroxylated on the β carbon to yield norepinephrine by dopamine β -hydroxylase (EC 1.14.17.1) within the vesicles. Dopamine β -hydroxylase knockout mice die during embryogenesis [31]. In the adrenal medulla and in a few brain regions, norepinephrine is converted to epinephrine by the enzyme phenylethanolamine *N*-methyltransferase.

The major mechanism by which the effects of norepinephrine are terminated is reuptake back into the nerve terminal by a high-affinity norepinephrine transporter (NET). Norepinephrine as well as epinephrine are metabolized to inactive products. Norepinephrine is metabolized by the enzymes monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) to 3-methoxy-4-hydroxyphenylglycol (MHPG), which in turn is oxidized to 3-methoxy-4-hydroxymandelic acid (VMA) by sequential actions of alcohol and aldehyde dehydrogenases [32]. The major metabolite found in the blood and urine is MHPG, which is incorrectly sometimes taken to provide an index of norepinephrine metabolism in the brain [32]. The majority of the metabolism of norepinephrine and epinephrine takes place in the same cells where the amines are produced. Importantly, most of this metabolism occurs independently of exocytotic release, and only a small fraction of the metabolites is formed from circulating norepinephrine and epinephrine [32].

The monoamine oxidases (EC 1.4.3.4; MAO) comprise a family of flavin adenine dinucleotide (FAD)-dependent enzymes with two members, MAO_A and MAO_B, which are products of distinct genes and have different substrate specificity [33]. The human MAO_A and MAO_B enzymes have 527 and 520 amino acid residues, respectively, resulting from their respective genes, each consisting of 15 exons. They catalyze the oxidative deamination of a variety of amines, including the catecholamines and serotonin neurotransmitters, trace amines (e.g., phenylethylamine and tyramine), and xenobiotic amines [33]. MAO_A [34] and MAO_B [35] knockout mice have been developed. In mice lacking MAO_A, norepinephrine concentrations were increased up to twofold and the mice manifested a distinct

behavioral syndrome, including enhanced aggression in males [34]. MAO_B-deficient mice show an increased reactivity to stress [35].

6.4 NOREPINEPHRINE TRANSPORTER

6.4.1 General Characteristics of NET

The NET is a member of a large family of Na⁺/Cl⁻-dependent transporters with submicromolar potency for substrates and is synthesized as a glycoprotein [36]. Other members of this family include dopamine, serotonin, γ -aminobutyric acid (GABA), glycine, proline, and taurine transporters [36, 37]. The human NET gene is located at chromosome 16q13-21 and consists of 15 exons which encode 617 amino acids [37]. It has 12 transmembrane domains and its conformation is similar to that of other membrane-associated proteins that are responsible for ion and solute transport (Fig. 6.5). The first 5 transmembrane domains of the amino terminal have been shown to be involved primarily in general uptake mechanisms. The next 3 transmembrane domains play a key role in determining tricyclic antidepressant and cocaine binding, while the carboxyl terminal region appears to mediate substrate interaction. NET concentrates norepinephrine (NE) by coupling its transport to cotransporter ions in a proposed stoichiometry of 1 NE : 1 Na⁺ : 1 Cl⁻ with a turnover rate of one transport cycle per second [38]. *N*-Glycosylation of NET appears to be essential for transporter assembly and surface expression but not for antagonist binding affinity [39].

The NET has a major role in terminating the neurochemical signal created by norepinephrine in the synaptic cleft. Reuptake of norepinephrine by NET (uptake 1) is the primary mechanism by which the biological effects of norepinephrine in the synapse are terminated. This axonal event is an energy-requiring, saturable, sodium- and chloride-dependent process.

Reuptake of norepinephrine is competitive with a variety of naturally occurring amines and drugs. Drugs of abuse such as amphetamine and cocaine and antidepressants (e.g., desipramine, imipramine, venlafaxine, reboxetine) block the

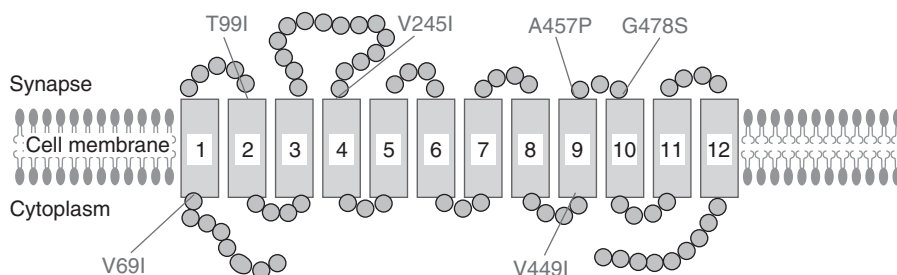


Figure 6.5 Molecular structure of NET protein. The NET protein consists of 617 amino acids and has 12 transmembrane domains (rectangles). This conformation is similar to that of other membrane-associated proteins that are responsible for ion and solute transport. Missense polymorphisms identified in the human NET gene are identified by lines. Note that the number of circles does not represent the number of amino acid residues. (Reprinted from [37a].) (See color insert.)

TABLE 6.1 Selectivity of Various Agents for NET

Drug	Binding			Transport		
	NET	SERT	Ratio	NET	5-HT	Ratio
	K _i (nM)	K _i (nM)	(SERT/NET)	K _i (nM)	K _i (nM)	(SERT/NET)
Reboxetine ^a	1.1	129	117	8	1,070	134
Desipramine	0.63	22	35	3.5	163	47
Nortripyline	1.8	15	8.3	21	279	13.3
Atomoxetine ^b	2	8.9	4.5	0.7	4.3	6.1
Amitriptyline	19	2.8	0.15	102	36	0.35
DDMI ^c	153	12.5	0.087			
Imipramine	20	1.3	0.065	142	20	0.141
Venlafaxine	2,269	7.5	0.0033	1,644	102	0.062
Fluoxetine	777	0.9	0.0012	2,186	20	0.0091
Fluvoxamine	2,950	1.6	0.0005	4,743	14	0.0030
Citalopram	7,865	1.5	0.0002	30,285	8.9	0.0003

Note: Values are the inhibition constants (K_i) for the human NET and serotonin transporter (SERT) [40]. Binding was done with [³H]nisoxetine (NET) and [³H]citalopram (SERT). Transport was done using [³H]norepinephrine and [³H]serotonin (5-HT).

^aRat [41a].

^bBinding [41]; transport, rat [41].

^cDesmethyldesimpramine, rat (J. D. Deupree and D. B. Bylund, unpublished data).

transport of norepinephrine. This results in an elevation of the synaptic concentrations of norepinephrine and potentiation of the activation of postsynaptic receptors [37, 40]. All of these agents have antidepressant activity and many are used clinically to treat depression [41]. The affinities of some of these agents for the NET as compared to the serotonin transporter are given in Table 6.1.

The NET is regulated during development [42] and by drugs that alter noradrenergic transmission, which causes changes in the sensitivity to endogenous catecholamines [43]. Approximately 80% of the norepinephrine released by sympathetic nerves is cleared by the NET, and the remaining percent spills over into the circulation and/or is cleared by extraneuronal tissue (uptake 2) [44]. Norepinephrine reuptake is particularly important in highly innervated tissues such as the heart.

6.4.2 Genetic Variations in Norepinephrine Transporter Gene

Several polymorphisms in the NET gene have been identified. A Taq1 restriction fragment length polymorphism was the first genetic variation identified in human NET [45]. A lack of linkage with major depression or bipolar disorder was reported, and this polymorphism has not been examined further. Alternative splicing from the human NET gene produces two variants designated *hNET C-t var1* and *hNET C-t var2* in addition to the typical *hNET* isoform. The differential characteristics of these isoforms may have functional consequences [46].

Mutations in the gene encoding the NET have been considered as a potential basis for some psychiatric illnesses. Thirteen NET DNA sequence variants have been identified in patients suffering from schizophrenia or bipolar affective disorder, although there was no evidence linking these polymorphisms to psychiatric illness [47]. The initial examination of a novel T-182C polymorphism in the promoter region of the NET gene indicated that this polymorphism was not associated with the susceptibility to major depression in a German population [48]. However, two subsequent studies by other investigators have suggested that the T-182C polymorphism might indeed be associated with major depression [49, 50].

6.5 α_1 -ADRENERGIC RECEPTORS

6.5.1 General Characteristics and Regulation of α_1 -Adrenergic Receptors

The α_1 -adrenergic receptors are single-polypeptide chains (446, 519, and 572 amino acid residues for the human α_{1A} , α_{1B} , and α_{1D} subtypes, respectively) that span the membrane seven times, with the amino terminal being extracellular and the carboxyl terminal intracellular. Thus, there are three intracellular loops and three extracellular loops. In contrast to the α_2 receptors but similar to the β receptors, the α_1 receptors have a long carboxyl terminal tail (137–179 amino acid residues) and a short third intracellular loop (68–73 amino acid residues). The amino terminal of the α_{1A} and α_{1B} subtypes have three (α_{1A}) or four (α_{1B}) consensus sites for N-linked glycosylation. The carboxyl terminal tails of all three subtypes are potentially palmitoylated, thus anchoring the tail to the membrane and forming a small fourth intracellular loop. The carboxyl terminal tails also have multiple sites of phosphorylation which are thought to be important in the desensitization, recycling, and downregulation of the receptor.

The human α_1 -adrenergic receptor gene consists of two exons and a single large intron of at least 20 kb in the region corresponding to the sixth transmembrane domain. No splice variants are known for the α_{1B} and α_{1D} subtypes. By contrast, at least 10 splice variants of human α_{1A} subtype have been reported, but only 4 produce full-length receptors. These three variants differ in the length and sequence of the C-terminal domains. Ligand binding and signal transduction do not appear to be altered by splicing of these full-length receptors. Splice variant 1 predominates in most human tissues and cell lines studied [51].

Adrenergic receptors, as well as other members of the seven-transmembrane receptor superfamily, have traditionally been thought to function as monomers, but a significant amount of recent evidence suggests that these receptors can also exist as dimers consisting of identical (homodimer) or distinct (heterodimer) subunits [52, 53]. Because most of the experimental techniques used to study dimerization cannot easily distinguish between dimers and larger oligomers, the term *dimer* is used for sake of simplicity. Dimerization of seven-transmembrane receptors may alter the functional, pharmacological, or regulatory properties of the receptor and, in some cases, may be absolutely required for receptor function. The α_{1A} adrenergic receptor splice variants produce both homo- and heterodimers [54] and α_{1B} receptors form homodimers [55]. The α_{1D} receptor has been shown to dimerize with both

TABLE 6.2 Examples of Adrenergic Drugs

	α_1 Adrenergic	α_2 Adrenergic	β Adrenergic
Agonists	Phenylephrine, methoxamine	Clonidine, brimonidine (UK14304)	Isoproterenol, terbutaline
Antagonists	Prazosin, terazosin, phentolamine	Yohimbine, RX821002, phentolamine	Propranolol, pindolol, metoprolol

α_{1B} - [56] and β_2 adrenergic [57] receptors. In both cases dimerization promotes the surface expression and functional activity of the α_{1D} receptor.

In addition to norepinephrine and epinephrine, α_1 receptors are activated by a variety of nonselective and subtype-selective agonists. Agents such as phenylephrine, methoxamine, and metaraminol are relatively selective for α_1 receptors and have little or no activity at α_2 and β receptors, except at high concentrations (Table 6.2). By contrast, they have similar affinities for the three α_1 subtypes and are thus considered to be non-subtype-selective agonists. Similarly, antagonists including prazosin, terazosin, doxazosin, and tamsulosin are relatively selective for α_1 receptors and block α_2 and β receptors only at high concentrations. These antagonists all have similar affinities for the three α_1 subtypes, although tamsulosin is considered to be slightly selective for the α_{1A} and α_{1D} subtypes as compared to the α_{1B} subtype. Several other antagonists such as phentolamine and phenoxybenzamine block both α_1 - and α_2 -adrenergic receptors with similar affinities.

Several agents have been developed that are at least 10- or 20-fold selective for the α_{1A} subtype as compared to the α_{1B} and α_{1D} subtypes [51]. However, agents that are clearly selective for either the α_{1B} or α_{1D} have not yet been developed, with the exception of BMY 7378, which is an α_{1D} -selective antagonist. The α_{1A} -selective antagonists include 5-methylurapidil, niguldipine, SNAP 5089, KMD 3213, RS 17053, and A131701 [51]. Potential α_{1A} -selective agonists include cirazoline, and A61603.

When adrenergic receptors are stimulated by an agonist, their subsequent responsiveness is decreased or blunted. This biological phenomenon is called desensitization or tachyphylaxis. Several different cellular processes, with different time courses, seem to be involved. These include modulation of receptor function (minutes), receptor internalization (sequestration and endocytosis; minutes to hours), recycling to the plasma membrane (hours), degradation (hours to days), and regulation of messenger ribonucleic acid (mRNA) levels (hours to days).

Two major types of desensitization have been identified. With homologous desensitization, reduced responsiveness is observed to the agonist that originally stimulated the receptor. In heterologous desensitization a decreased responsiveness is observed with an agent unrelated to the initial stimulus. It is generally accepted that homologous desensitization involves receptor phosphorylation by a GPCR kinase of the agonist-occupied receptor, resulting in the binding of β -arrestin [58, 59].

The α_{1B} -adrenergic receptor has been the most studied of the α_1 subtypes with respect to desensitization [59]. Agonist stimulation results in the phosphorylation of specific sites located in the carboxyl tail of the receptor (serines 404, 408, and 410) which is associated with desensitization and internalization. Direct activation of protein kinase C leads to α_{1B} receptor desensitization and internalization associated

with phosphorylation by protein kinase C (serines 394 and 400). Activation of other G_q -coupled receptors as well as G_i -coupled receptors can also induce α_{1B} receptor desensitization and involves phosphorylation by protein kinase C [59, 60]. It appears that the α_{1A} [61] and the α_{1D} [62] subtypes also undergo desensitization that involves phosphorylation, although the evidence is less complete.

Transcriptional regulation of α_1 -adrenergic receptors has been shown to occur under a variety of conditions, including hypoxia, ischemic reperfusion, agonist stimulation, cyclic adenosine monophosphate (AMP) levels and growth factors [60]. The α_{1B} gene is upregulated under hypoxic conditions in vascular smooth muscle cells, whereas the α_{1D} receptor is selectively downregulated (both mRNA and protein) by platelet-derived growth factor-BB in this same cell type. Stimulation of neonatal rat cardiomyocytes with norepinephrine leads to an increase in α_{1A} message levels, whereas α_{1B} and α_{1D} levels are reduced. Several other agents that also cause hypertrophy, including phorbol esters endothelin-1 and prostaglandin F₂, as well as aortic banding in the whole animal (creating hypertrophy through pressure overload) cause similar effects [63].

6.5.2 Signal Transduction Pathways for α_1 -Adrenergic Receptors

All three of the α_1 -adrenergic receptor subtypes activate the $G_{q/11}$ family of G proteins leading to the dissociation of the α and $\beta\gamma$ subunits and the subsequent stimulation of the enzyme phospholipase C. This enzyme hydrolyzes phosphatidylinositol in the membrane producing inositol trisphosphate (IP₃) and diacylglycerol. These molecules act as second messengers mediating intracellular Ca^{2+} release via the IP₃ receptor and activating protein kinase C. Other signaling pathways that have also been shown to be activated by α_1 receptors include Ca^{2+} influx via voltage-dependent and voltage-independent calcium channels, arachidonic acid release, and activation of phospholipase A₂, phospholipase D activation, and mitogen-activated protein kinase (MAPK) [51, 64, 65]. In general, the α_{1A} subtype appears to couple most efficiently to intracellular signaling cascades whereas the α_{1D} couples least efficiently [66]. All three subtypes activate $G_{q/11}$, but only the α_{1A} and α_{1B} subtypes couple to G_{14} , and only α_{1B} couples to G_{16} [67]. The α_{1B} receptors (but not the other two) can also couple to G_o .

In addition to regulating calcium movements and smooth muscle contraction, the α_1 -adrenergic receptors also help regulate cell proliferation through the three major subfamilies of the MAPK family [extracellular regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and the p38 kinases]. The activation of the various MAPK members appears to be highly dependent on the particular α_1 receptor subtype(s) present and also on the particular cell line or tissue being studied [51, 65].

6.5.3 Physiological Roles for α_1 -Adrenergic Receptors

In the periphery, a primary function of α_1 -adrenergic receptors appears to be in maintaining resting vascular tone. This is due primarily to the presence of postjunctional α_1 receptors in a wide array of vascular beds in the systemic arterial circulation. By contrast, in the venous system both α_1 and α_2 receptors contribute to the vasoconstriction [64]. The question of which α_1 subtype is involved in vasoconstriction is not easy to determine because most vascular smooth muscle

tissues express mixtures of α_1 subtypes. Thus, in most cases responses to α_1 agonists are probably due to activation of more than one receptor subtype. For example, in the rat the α_{1A} and α_{1D} subtypes appear to regulate the larger vessels, whereas the α_{1B} subtype controls the small resistance vessels [64]. Recent studies with knockout mice also indicate that all subtypes play a role in the blood pressure response to α_1 agonists through their vasocontractile responses. The extent of the contribution of each of the α_1 subtypes to the contraction of vascular smooth muscle involved has not yet been completely resolved by these studies [68]. In addition, α_1 receptors may also be involved in the regulation of vascular smooth muscle growth; the α_{1B} receptor appears to induce hypertrophy of arterial smooth muscle cells, whereas the α_{1A} attenuates this growth response [64].

Selective α_{1A} -adrenergic receptor antagonists appear to have significant therapeutic advantages over non-subtype-selective α_1 antagonists in the treatment of benign prostatic hypertrophy. Although the α_{1A} subtype is the predominant α_1 subtype in human prostatic smooth muscle and does mediate contraction in this tissue, functional studies have not yet shown that this is the primary subtype mediating the beneficial response [64].

Although the predominant postjunctional adrenergic receptor type in the heart is the β -adrenergic receptor, the α_1 receptors play an important subsidiary role, particularly in increasing the force of contraction at low rates of beating. This inotropic effect appears to be mediated by both the α_{1A} and α_{1B} subtypes [69, 70]. The α_{1A} subtype also appears to be the subtype primarily responsible for the events leading to cardiac hypertrophy caused by adrenergic agents [70].

The role of the α_1 receptors in CNS function is only poorly understood. They appear to be postsynaptic, stimulatory receptors and affect many brain functions, at least in part through nonneuronal mechanisms because they are also present in glial cells [68]. The α_{1B} subtype predominates in the cortex in both the human and rat brain. Recent data suggest that the α_1 receptors are involved in locomotion and the control of motor activity as well as in cognitive functions and behaviors such as learning, memory, and fear. Interestingly, the α_{1B} subtype may enhance apoptotic neurodegeneration, which suggests that α_{1B} antagonists may have therapeutic potential in the treatment of human neurodegenerative disorders [68].

Several α_1 -adrenergic receptor polymorphisms have been noted, including a C-to-T polymorphism in the α_{1A} subtype. No linkage, however, has been found between this polymorphism and attention-deficit hyperactivity disorder, Alzheimer's disease, or the reward-dependence personality trait.

6.6 α_2 -ADRENERGIC RECEPTORS

6.6.1 General Characteristics and Regulation of α_2 -Adrenergic Receptors

The α_2 -adrenergic receptors are single-polypeptide chains (450, 450, and 461 amino acid residues for the human α_{2A} , α_{2B} , and α_{2C} subtypes, respectively) that span the membrane seven times, with the amino terminal being extracellular and the carboxyl terminal intracellular. In contrast to the α_1 and β receptors, the α_2 receptors tend to have long third intracellular loops (148–179 amino acid residues) and a short carboxyl terminal tail (20–21 amino acid residues). The amino terminal of the α_{2A} and α_{2C}

subtypes have two consensus sites for N-linked glycosylation, and the carboxyl terminal tails of all three subtypes are potentially palmitoylated, thus anchoring the tail to the membrane and forming a fourth intracellular loop. The third intracellular loops have multiple sites of phosphorylation which are thought to be important in the desensitization, recycling, and downregulation of the receptor. The α_2 -adrenergic receptor genes do not contain introns, and thus there are no splice variants.

In addition to norepinephrine and epinephrine, α_2 receptors are activated by several nonselective and subtype-selective agonists. Agents such as clonidine, guanfacine, guanabenz, and brimonidine are relatively selective for α_2 receptors and have lower affinity at α_1 and β receptors (Table 6.2). By contrast, they have similar affinities for the three α_2 subtypes and are thus considered to be non-subtype-selective agonists. Similarly, antagonists including yohimbine, idazoxan, and RX821002 are relatively selective for α_2 receptors and block α_1 and β receptors only at higher concentrations. These antagonists have similar affinities for the three α_2 subtypes. Antagonists that are at least somewhat selective for one of the α_2 subtypes include BRL44408 for the α_{2A} , prazosin and ARC-239 for the α_{2B} (note, however, that these two agents have much higher affinities for α_1 receptors), and rauwolscine for the α_{2C} subtype [71]. Ozymetazoline is a partial agonist that has a higher affinity for the α_{2A} subtype as compared to the α_{2B} and α_{2C} subtypes.

Most α_2 -adrenergic receptors also undergo desensitization and downregulation (a decrease in receptor density) following stimulation by epinephrine, norepinephrine, or other α_2 agonists, as described above for the α_1 receptors. There are, however, subtype, tissue, and species differences in the extent of desensitization [72]. Desensitization after short-term agonist exposure appears to involve G-protein-coupled receptor kinase (GRK)-dependent phosphorylation of sites in the third intracellular loop of the human α_{2A} and α_{2B} receptors. In contrast, the human α_{2C} receptor does not desensitize, although the opossum α_{2C} receptor does desensitize [73, 74]. Agonist-induced downregulation in response to long-term (~ 24 -h) epinephrine or norepinephrine exposure occurs for all three subtypes in cell lines normally expressing the receptor as well as in cell lines transfected with the receptors. The time course of downregulation varies with subtype, species, and cell type. In cell lines, it appears that an increase in the rate of receptor degradation is the mechanism for downregulation of α_{2A} and α_{2B} receptors [75]. A common polymorphism has been identified in the third intracellular loop of the α_{2B} (a deletion of three glutamate residues) resulting in a loss of short-term agonist-induced desensitization [76].

Receptor regulation appears to be important in the etiology and treatment of clinical depression. A characteristic of most antidepressant drugs is that they increase the synaptic concentration of norepinephrine (and other monoamines) by inhibiting metabolism (MAO inhibitors), blocking the high-affinity reuptake (classical tricyclic antidepressants, selective norepinephrine reuptake inhibitors) or blocking the pre-synaptic α_2 -adrenergic inhibition of neurotransmitter release (α_2 antagonists). Thus, the mechanisms of downregulation produced by these drugs are thought to be similar to those of agonist-induced downregulation. In the rat brain α_2 -adrenergic receptors are desensitized and downregulated following chronic, but not acute, administration of antidepressants [77–79]. Recent evidence from knockout mice suggests that the effects of the antidepressant imipramine are mediated by the α_{2A} receptor subtype [80]. Except for the caudate (or striatum in the rat) the predominant subtype is the α_{2A} (or α_{2D} in the rat) in nearly all brain areas [81].

During postnatal development there is an overall increase in α_2 receptor levels with great variability in pattern and timing of receptor density changes among brain regions. Three major patterns are apparent. First, in many regions receptor density increases during postnatal development, generally reaching adult levels around postnatal day 15. Second, there are regions with very high levels of receptors at birth and little or no change in density during the postnatal period. Third, in some regions α_2 -adrenergic receptors either decrease or are only transiently expressed during the course of postnatal development, including white matter regions, cerebellum, and many brain stem nuclei, suggesting specific roles for α_2 receptors during development [82].

Using agonist-stimulated [35 S]GTP γ S binding as a functional assay, it has been shown that α_2 -adrenergic receptors are coupled to G proteins throughout postnatal development [83].

6.6.2 Signal Transduction Pathways for α_2 -Adrenergic Receptors

All three of the α_2 -adrenergic receptor subtypes activate the $G_{i/o}$ family of G proteins leading to the dissociation of the α and $\beta\gamma$ subunits and a subsequent alteration (classically inhibition) in the activity of the enzyme adenylate cyclase, which lead to changes in the concentration of the second messenger cyclic AMP. In addition, the stimulation of α_2 receptors can affect several other effector systems, including the activation of K^+ channels, inhibition or activation of Ca^{2+} channels, and activation of phospholipase A_2 , phospholipase C, and Na^+/H^+ exchange [84].

The regulation of adenylate cyclase by receptors that activate the G_i family of G proteins is complex due the existence of nine differentially regulated isoforms (or isoforms), each the product of a different gene [85]. The α_i subunit inhibits the activity of isoforms 5 and 6 by acting at a site symmetrical to the G_s binding site (which stimulates enzyme activity). In addition, the released $\beta\gamma$ subunits are strong modulators of adenylate cyclase activity and can either be stimulatory (at isoforms 2, 4, and 7) or inhibitory (at isoforms 1 and 8). In fact, the $\beta\gamma$ subunit is among the most potent of all negative regulators of isoforms 1 and 8 and can markedly inhibit the enzyme activity stimulated by forskolin, G_s , and Ca^{2+} -calmodulin. This is particularly relevant to the functioning of the CNS because both the G_i proteins and cyclase isoforms 1 and 8 are highly expressed in the brain [85]. Released $\beta\gamma$ subunits stimulate the cyclase activity of isoforms 2, 4, and 7, but only when adenylate cyclase has already been activated by G_s . This synergistic activation of cyclase by receptors coupled to G_i and by receptors coupled to G_s has been termed "coincidence signaling" and may play a prominent role in the integration of the various signals received by a given neuron.

The α_2 -adrenergic activation of G_i -protein-gated K^+ channels results in membrane hyperpolarization, which in turn causes a decrease in the firing rate of excitable cells in the CNS. This effect is Ca^{2+} dependent in many but not all systems [84]. Decreased Ca^{2+} conductance mediates α_2 -adrenergic inhibition of neurotransmitter release, one of the most prominent physiological actions of α_2 receptors. This effect involves direct inhibition of N-type voltage-gated Ca^{2+} channels and can be independent of cyclic AMP and protein phosphorylation [84].

6.6.3 Physiological Roles for α_2 -Adrenergic Receptors

The α_2 -adrenergic receptors, which are found throughout the peripheral and central nervous systems, mediate a variety of effects including hypotension, sedation, and antinociception as well as a variety of behavioral effects. In addition, α_{2A} receptors on platelets mediate aggregation and stimulation of α_2 receptors in the pancreas inhibits insulin release.

Activation of α_{2A} receptors in the rostral ventrolateral medulla decreases sympathetic outflow, causing a reduction in arterial blood pressure and heart rate [86]. This mechanism is the means by which α_2 agonists such as clonidine lower blood pressure in hypertensive patients. In addition to this centrally mediated response, there is a transient hypertensive response following rapid intravenous (i.v.) injection, caused mainly by α_{2B} -adrenergic receptor-mediated vasoconstriction of vascular smooth muscle [87, 88]. The α_2 subtype mediating these effects has been determined through the use of mice lacking each of the subtypes (knockout animals). The hypotensive response to administration of α_2 agonists was abolished in α_{2A} knockout animals, demonstrating that the α_{2A} subtype plays a principal role in this response. The bradycardic response to agonist was also blunted [89]. The hypertensive response was abolished in α_{2B} knockout mice, and the hypotensive effect was immediate and accentuated. The bradycardic response in α_{2B} knockout mice was normal, and α_{2C} knockout mice showed no differences from wild-type strains in their hypertensive, hypotensive, and bradycardic effects [87, 88].

Due to the sedative effects mediated by α_{2A} -adrenergic receptors, α_2 agonists have been used as anesthetic agents in veterinary practice for the past 25 years and are now finding use in humans as adjuncts to anesthesia [90]. The sedative effects of α_2 agonists were abolished in mice lacking the α_{2A} subtype as determined by rotarod, loss of righting reflex and spontaneous locomotor activity tests [91], indicating that the α_{2A} -adrenergic receptor mediates the sedative effects of agonist administration [87, 88]. In contrast, both α_{2B} and α_{2C} knockout mice show dose-dependent sedative effects which are indistinguishable from wild-type mice. α_2 agonists appear to induce sedation by activating presynaptic autoreceptors in the locus ceruleus, reducing its spontaneous rate of firing. In addition, α_2 agonists can reduce the requirement for other anesthetic agents by as much as 90% [90]. This anesthetic-sparing effect also appears to be mediated by the α_{2A} subtype.

Another therapeutic effect mediated by the α_{2A} -adrenergic receptor is analgesia. The α_2 -adrenergic agonists have analgesic properties when given parenterally, epidurally, or intrathecally. Descending noradrenergic antinociceptive systems originating in the brain stem contribute to pain control by suppressing the spinal centripetal transmission of nociceptive impulses [90]. Once again the α_{2A} subtype appears to mediate this effect, based on studies in genetically engineered mice using the ramped hot plate, hot-water immersion, and tail-flick latency tests [87, 88]. Similar to the hypertensive, sedative, and analgesic effects, the hypothermic effect also appears to be mediated primarily by the α_{2A} subtype with a small contribution from the α_{2C} subtype. The α_{2A} -adrenergic receptor also mediates the antiepileptogenic actions of norepinephrine in the kindling model of epileptogenesis [87].

The α_{2A} -adrenergic receptor also appears to be the subtype mediating the effects of antidepressant drugs in the mouse [80]. A genetic knockout of the α_{2A} receptor makes

mice less active in the forced-swim test and insensitive to the antidepressant effects of the tricyclic drug imipramine. Thus, the α_{2A} -adrenergic receptor may play a protective role in some forms of human depression and anxiety and the antidepressant effects of imipramine and other antidepressants may be mediated in part by the α_{2A} -adrenergic receptor.

Presynaptic inhibition of norepinephrine release is a classical α_2 -adrenergic receptor function. The α_2 agonists can inhibit release of norepinephrine from nerve terminals and antagonists can enhance release. These negative-feedback effects are mediated by terminal and by somadendritic α_2 receptors [92]. There is, however, some debate as to whether this extends to endogenously released norepinephrine. Some authors question if under normal physiological conditions there is local regulation of neuronal transmitter release by autoreceptors as a result of these receptors being activated by the perineuronal concentration of previously released transmitter [93]. Initial data from knockout animals indicated that the α_{2A} subtype was the most important in mediating presynaptic α_2 -adrenergic receptor inhibition of neurotransmitter release, although a role for at least one other subtype seemed probable. Subsequent studies on the sympathetic nerves in the hearts of mice lacking one or both (double knockout) of the α_{2A} and the α_{2C} subtypes indicated that the α_{2A} receptor inhibits transmitter release at high stimulation frequencies, whereas the α_{2C} subtype regulates release at lower levels. The regulation at both high and low frequencies appears to be physiologically important [87, 88].

In comparison to the α_{2A} subtype, relatively less has been discovered about the functions of the α_{2B} and α_{2C} subtypes through knockout experiments. As noted above, the α_{2B} subtype appears to have a dominant role in eliciting the vasoconstrictor response to α_2 -adrenergic agonists, at least under some situations. The α_{2B} -adrenergic receptor has also been implicated in salt-induced hypertension and may be important in developmental processes, although the actual role it plays is currently unknown. The α_2 receptors are expressed on vascular smooth muscle cells and initiate vasoconstriction, although they have a unique distribution in the human vasculature as compared to α_1 receptors [64]. Because of their role in vascular thermoregulation, α_2 -adrenergic receptors are more active in cutaneous as compared with deep blood vessels [94]. They also have considerable activity in blood vessels of the nasal mucosa, where their activity predominates over α_1 -adrenergic receptors [95, 96]. Indeed, phenylpropanolamine (*dl*-norephedrine), once widely used as a nasal decongestant, is a preferential α_2 -adrenergic agonist [97].

The α_{2C} subtype does not appear to play a significant role in cardiovascular regulation or the other classical effects of α_2 -adrenergic receptors, with the exceptions of the hypothermic and presynaptic effects noted above. Studies with genetically modified mice suggest that the α_{2C} subtype may play a role in stress-dependent depression, in startle responses and prepulse inhibition (which may relate to schizophrenia, attention-deficit hyperactivity disorder, and posttraumatic stress disorder), in modulating motor behavior, and perhaps in memory processes [87, 88].

The α_{2C} receptor has a higher density in the striatum compared to other brain regions, but its physiological role is unknown. Recent studies with knockout mice suggest that the α_{2C} subtype regulates striatal GABA release and that the endogenous catecholamine activating the receptor may be dopamine rather than norepinephrine [98].

The α_{2C} subtype may also play a role in modulation of schizophrenia symptoms by antipsychotic drugs. Although the antipsychotic agents display rather equivalent efficacy against the positive symptoms of schizophrenia, they differ with respect to improvement of the negative symptoms such as cognitive dysfunction. These differences are potentially related to the relative affinities of the antipsychotics for the dopamine D_2 receptor and for receptors for other neurotransmitter systems. Interestingly, the affinity of clozapine, one of the so-called atypical antipsychotics, is about 12 times more potent at the α_{2C} receptor than at the dopamine D_2 receptor, suggesting that α_{2C} -adrenergic receptor blockade may contribute to the improvement of cognitive function produced by clozapine [99].

Polymorphisms have been identified in each of the α_2 -adrenergic receptor subtypes. For the α_{2A} subtype, there are two known polymorphisms in the promoter region (identified by Msp-I and Dra-I). No evidence was found for linkage of the α_{2A} gene with attention-deficit hyperactivity disorder. In addition, there is an infrequent polymorphism (lysine for asparagine at amino acid residue 252) that results in enhanced G_i coupling and a six-residue (255–260) deletion [100].

A common polymorphism has been identified in the third intracellular loop of the α_{2B} receptor, which consists of a deletion of three glutamate residues (301–303) and is a risk factor for acute coronary events but not hypertension. This deletion results in a loss of short-term agonist-induced desensitization [100].

For the α_{2C} subtype, a common polymorphism has been identified in the third intracellular loop which consists of a deletion of four amino acid residues (322–325) and results in an impaired coupling to several effectors [100]. No evidence for linkage of a dinucleotide repeat polymorphism located approximately 6 kb from the gene was found with attention-deficit hyperactivity disorder [101].

6.7 β -ADRENERGIC RECEPTORS

6.7.1 General Characteristics and Regulation of β -Adrenergic Receptors

The β -adrenergic receptors are single-polypeptide chains (477, 413, and 408 amino acid residues for the human β_1 , β_2 , and β_3 subtypes, respectively) that span the membrane seven times, with the amino terminal being extracellular and the carboxyl terminal intracellular. In contrast to the α_2 receptors but similar to the α_1 receptors, the β receptors tend to have longer carboxyl terminal tails (61–97 amino acid residues) and shorter third intracellular loops (54–80 amino acid residues). The amino terminal of the β receptors have one or two consensus sites for N-linked glycosylation, and the carboxyl terminal tails of all three subtypes are potentially palmitoylated, thus anchoring the tail to the membrane and forming a small fourth intracellular loop. The carboxyl terminal tails also have multiple sites of phosphorylation which are thought to be important in the desensitization, recycling, and downregulation of the receptor.

The β_1 - and β_2 -adrenergic receptor genes do not contain introns, and thus they have no splice variants. By contrast, the β_3 receptor has one intron, resulting in two splice variants. However, no functional differences have been found between the splice variants [12].

The β_2 -adrenergic receptors have been shown to form homodimers [102, 103] as well as heterodimers with opioid [104] and α_{1D} -adrenergic [57] receptors. These associations appear to have important functional consequences in terms of trafficking and signaling activity.

In addition to norepinephrine and epinephrine, β -adrenergic receptors are activated by a variety of nonselective and subtype-selective agonists. Isoproterenol is the prototypic non-subtype-selective β agonist which has no activity at α_1 and α_2 receptors, except at high concentrations (Table 6.2). Epinephrine is 10- to 100-fold more potent at the β_2 receptor as compared to the β_1 subtype, whereas norepinephrine is more potent than epinephrine at the β_3 subtype. Many β_2 -selective agonists have been developed for the treatment of asthma. Due to their subtype selectivity they have a lower incidence of side effects mediated by the β_1 receptor. These β_2 -selective agonists include metaproterenol, terbutaline, albuterol, salmeterol, and ritodrine. A relatively selective agonist for the β_3 receptor is BRL37344 [11].

Propranolol is the prototypic non-subtype-selective β antagonist which has equal affinities at the β_1 and β_2 subtypes and no activity at α_1 and α_2 receptors, except at high concentrations. Other nonselective β -adrenergic antagonists include nadolol, timolol, pindolol (which is actually a weak partial agonist), and carvedilol, which is also an α_1 antagonist. Propranolol, as well as other classical β antagonists, has low affinity for the β_3 subtype, which has previously been referred to as the atypical β receptor [71]. Several β_1 -selective antagonists have been developed, such as metoprolol, esmolol, acebutolol, and CGP 20712. A useful β_2 -selective antagonist is ICI 118551 [71].

The processes involved in desensitization have been extensively investigated for the β_2 -adrenergic receptor, which is the prototypic GPCR model with cells in culture. Initial uncoupling of the β_2 receptor from the G protein after agonist binding is mediated by phosphorylation of specific residues in the carboxyl tail of the receptor. The phosphorylated β_2 receptor serves as a substrate for the binding of β -arrestin, which not only uncouples the receptor from the signal transduction process but also serves as an adapter protein that mediates the binding of additional signaling proteins and entry into the internalization pathway [105, 106]. The mechanisms of β_2 -adrenergic receptor downregulation appear to involve both an increase in the rate of degradation of the receptor and a decrease in the levels of β receptor mRNA [107].

A common result of chronic but not acute antidepressant drug administration to rats is downregulation of β -adrenergic receptors and/or desensitization of β -adrenergic-stimulated adenylate cyclase. This has been observed with most classes of antidepressant drugs as well as with electroconvulsive shock [108, 109]. This downregulation is not immediate but occurs only after days to weeks of drug treatment, consistent with the therapeutic time course. Downregulation is more likely to be observed with the β_1 - as compared to the β_2 -adrenergic receptor [110].

6.7.2 Signal Transduction Pathways for β -Adrenergic Receptors

All three of the β -adrenergic receptor subtypes activate the G_s family of G proteins leading to the dissociation of the α and $\beta\gamma$ subunits and subsequent activation of the enzyme adenylate cyclase, which lead to increases in the concentration of the second messenger cyclic AMP. In addition to G proteins, β -adrenergic receptors interact with many other signaling proteins. For example, the cytoplasmic tail of the

β_2 -adrenergic receptor binds to the phosphoprotein EBP50 (ezrinradixin-moesin-binding phosphoprotein-50) through a PDZ domain and to the Na^+/H^+ exchanger regulatory factor (NHERF) also through a PDZ domain [111], whereas the β_1 -adrenergic receptor binds to CNrasGEF (cyclic AMP-dependent Ras exchange factor), which leads to Ras activation [112]. The identification and functional significance of these interactions is currently an area of intense study [111]. Following binding of agonist, the β_2 receptor is phosphorylated by β -adrenergic receptor kinase (β ARK) and the phosphorylated receptor binds β -arrestin [105, 106]. The β -arrestin serves as an adapter or scaffolding protein, which binds a variety of other proteins such as clathrin (important in the internalization of the receptor), and various signaling proteins including Src family tyrosine kinases and the ERK and JNK MAPKs [106, 111].

6.7.3 Physiological Roles for β -Adrenergic Receptors

The β -adrenergic receptors modulate a wide range of physiological responses in the intact animal, including cardiac contraction, vascular and smooth muscle tone, and carbohydrate and lipid metabolism. The β_1 subtype is considered to be the “cardiac” β receptor because these receptors predominate in the heart and in vivo stimulation of these receptors with agonists increases both force and rate of cardiac contraction. The β_2 -adrenergic receptors are also expressed in the heart and in some species influence heart rate and contractility [113]. There is considerable heterogeneity in the tissue distribution and function of β receptor subtypes in the heart. In the adult human left ventricle, the ratio of β_1 to β_2 receptors is 80 : 20, whereas in the atria the ratio decreases to 70 : 30 [114]. The β_2 -adrenergic receptor is the predominant subtype mediating peripheral vascular as well as bronchial and uterine smooth muscle relaxation. Thus, β_2 agonists are useful in the treatment of asthma and premature labor. There are also differences in β -adrenergic signaling during development, and cardiac β_2 -adrenergic receptors may play a more substantial role in mediating contractile changes in the noninnervated fetal and neonatal heart [113].

In the CNS, β_1 -adrenergic receptors are generally associated with forebrain structures such as the cerebral cortex, striatum, and hippocampus, whereas the β_2 subtype predominates in the cerebellum, as determined by radioligand binding, in situ hybridization, and immunohistochemistry [115]. There is some evidence that indicates that a significant fraction of the β -adrenergic receptors may be located on glial cells.

A decrease in norepinephrine-stimulated cyclic AMP accumulation in the cortex is observed following chronic (but not acute) treatment with a variety of typical and atypical antidepressant agents as well as for monoamine oxidase inhibitors and electroconvulsive shock. Selective serotonin reuptake inhibitors such as zimelidine and clomipramine as well as norepinephrine reuptake inhibitors reduce norepinephrine-stimulated adenylate cyclase activity. Most but not all antidepressant treatments also downregulate β -adrenergic receptor density in many brain regions. In spite of intense research, the role played by β -adrenergic receptors in depression and in the mechanism of antidepressant drugs is still not well understood [116].

Gene knockout approaches have been utilized to disrupt expression of all three β -adrenergic receptors individually as well as in various (double-knockout) combinations. The role of each of the subtypes regulating cardiovascular physiology and

metabolism has then been determined in these mice [113]. Neither the β_1 nor the β_2 subtype is required for normal cardiac development. Mice lacking the β_1 , the β_2 , or both subtypes have normal resting heart rate, blood pressure, and cardiac output, suggesting that although these β -adrenergic receptors are responsible for regulating changes in heart rate, blood pressure, and contractility during activity or stress, they are not required for maintaining normal function [113]. The β_1 -adrenergic receptor is primarily responsible for sympathetically mediated changes in heart rate during stress or exercise. In the CNS, the cortex contains mostly β_1 receptors, whereas the cerebellum has a higher density of β_2 receptors as compared to other brain regions.

The gene encoding the human β_1 -adrenergic receptor is quite polymorphic with 18 single-nucleotide polymorphisms (SNPs), 17 within the coding exon for the receptor, 7 leading to amino acid substitutions, and it has been proposed that there may be 11 different genotypes [117]. The two amino acid substitutions of most interest are glycine for serine at amino acid residue 49 and arginine for glycine at residue 389. The latter polymorphism variant shows a gain of function phenotype in that it has an enhanced coupling to G_s and an increased isoproterenol-stimulated cyclic AMP response [117]. The other consistently reported alteration is an enhanced susceptibility to agonist-promoted downregulation of the glycine-49 genotype. The current data suggest an enhanced function of those two alleles, but the magnitude of the enhancement does not appear to be sufficient to allow consistent detection [117].

A total of 13 polymorphisms in the β_2 -adrenergic receptor gene and its transcriptional regulator upstream peptide have been identified [118]. Three closely linked polymorphisms, two coding regions at amino acid positions 16 and 27 and one in the upstream peptide, are common (i.e., allele frequency >0.15) in the general white population. The glycine-16 receptor exhibits enhanced downregulation *in vitro* after agonist exposure. In contrast, arginine-16 receptors are more resistant to downregulation [118]. Although initial studies suggested a relationship between the glycine-16 polymorphism and increased risk of severe asthma and increased airway responsiveness, subsequent associations with clinical asthma, atopy, and airway responsiveness have been inconsistent [119]. Liggett [118] has proposed a dynamic model of receptor kinetics to explain the polymorphism data. Glycine-16/glycine-16 homozygous individuals are already downregulated as a result of exposure to endogenous catecholamines. Thus, the tachyphylaxis caused by recurring exogenous exposure to a β -adrenergic agonist would be more apparent in the arginine-16/arginine-16 patients because their receptors had not yet been downregulated. In this model, the initial response to albuterol in β -agonist-naïve patients would be depressed in those who are glycine-16/glycine-16 homozygous because their receptors had been endogenously downregulated to a greater extent than the receptors of patients who are arginine-16/arginine-16 [118].

The β_3 -adrenergic receptor agonists are very effective thermogenic antiobesity and antidiabetic (insulin-sensitizing) agents in rodents. Their main sites of action are white and brown adipose tissue and muscle. Although adult humans have little brown adipose tissue, β_3 receptors are expressed in human white as well as brown adipose tissue and in skeletal muscle, and they play a role in the regulation of energy balance and glucose homeostasis [120]. Early evidence for the β_3 -adrenergic receptor stemmed from the discovery that β_1 and β_2 receptor antagonists lacked potency in various gut preparations and as antagonists of β -agonist-driven lipolysis. Novel β agonists were then discovered which were more potent as stimulants of lipolysis than

of atrial contraction. These compounds were found to stimulate metabolic rate and to have antiobesity and antidiabetic activity in rodents [120]. Although β_3 is the major β subtype controlling lipolysis, it also plays a role in regulating vascular tone cardiac contractility [113].

Targeted disruption of the mouse β_3 -adrenergic receptor creates conditions that predispose the animal to the development of obesity [121]. Somewhat surprisingly, however, neither mice lacking any of the three β receptors nor mice lacking both the β_1 and β_2 subtypes (double knockout) are cold sensitive or become overtly obese. By contrast, triple-knockout mice (β_1 , β_2 and β_3 all deleted) exhibit obesity and cold intolerance [122]. Thus, β -adrenergic signaling through at least one of the subtypes is essential for the resistance to obesity and cold.

A tryptophan-64-to-arginine polymorphism has been identified in the β_3 -adrenergic receptor. The allele frequency is approximately 30% in the Japanese population, higher in Pima Indians, and lower in Caucasians [123]. Type 2 diabetic patients with this mutation showed a significantly younger onset age of diabetes and an increased tendency to obesity, hyperinsulinemia, and hypertension [123].

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DOPAMINERGIC NEUROTRANSMISSION*

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7.1	Introduction	221
7.2	Chemistry and Metabolism of DA	222
7.3	Historical Classification and Molecular Properties of DA Receptors	225
7.3.1	The D ₁ Receptor Subfamily	225
7.3.2	The D ₂ Receptor Subfamily	227
7.4	DA Receptors as Signal Transducers	230
7.5	Molecular Pharmacology of DA Receptors: Structure–Affinity and Structure–Activity Relationships	232
7.6	Splice Variants, SNPs, and other Polymorphisms with Functional Consequences	234
7.7	Classification of Dopaminergic Drugs According to Treatment Category	236
7.7.1	Parkinson’s Disease	236
7.7.2	Schizophrenia	237
7.7.3	Bipolar Mania, Autism, Alzheimer’s Disease, and Tourette’s Syndrome	237
7.7.4	Attention-Deficit Hyperactivity Disorder	237
7.7.5	Substance Use	238
7.7.6	Other Uses	238
	References	239

7.1 INTRODUCTION

Dopamine (DA) is a neurotransmitter in the central and peripheral nervous systems where it regulates numerous physiological processes. Within the central nervous system (CNS), dopamine is known to regulate emotion, reward, cognition, memory, endocrine functions, and motor control. Alterations in dopaminergic transmission are known to be involved in the etiology and/or therapy of a number of neurological and psychiatric disorders, including Parkinson’s disease, Tourette’s syndrome,

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attention-deficit hyperactivity disorder (ADHD), schizophrenia, and substance use. One of the hallmarks of these disorders is that they are all treated with drugs that either enhance or impede dopaminergic transmission.

7.2 CHEMISTRY AND METABOLISM OF DA

Dopamine is among the most ancient of extant molecules innate to biological systems. It is a component of some plants [1–7] and is broadly distributed across the animal kingdom [8, 9]. Dopamine is a member of the general chemical class of compounds known as phenylethylamines, monoamines, and biogenic amines. The International Union of Pure and Applied Chemistry (IUPAC) and common chemical names for dopamine are 4-(2-aminoethyl)benzene-1,2-diol, 3,4-dihydroxyphenethylamine, 3-hydroxytyramine, and 2-(3,4-dihydroxyphenyl)ethylamine. Its chemical structure is comprised of a catechol ring (3,4-dihydroxyphenyl) linked by an ethyl to a primary amine: Dopamine has a catecholamine pharmacophore (Fig. 7.1). The presence of ionizable hydroxyl and amine groups at physiological pH and DA's small molecular size all contribute to its high water solubility. This restricts its distribution to the aqueous phase and prevents it from readily traversing biological lipid barriers and thus restricts its passive transport across blood–gut and blood–brain barriers. Although the mechanism by which DA is actively transported across biological barriers is not well understood, DA is a good substrate for the norepinephrine transporter [11, 12] and this transporter is localized in capillary endothelial cells at the blood–brain barrier [13]. However, the DA precursor dihydrophenylalanine (L-DOPA) is actively transported by the neutral bulky amino acid transport complex 4F2hc/LAT1 that is situated in cellular membranes comprising the blood–brain barrier, which may explain why lower plasma levels of orally administered L-DOPA are observed following a high-protein meal [14, 15].

The catechol moiety of DA makes it susceptible to oxidation. Under oxidative conditions, the catechol is converted to a highly reactive quinone (3,4-diketophenyl) which, through a series of reactions, is converted to water-insoluble conjugated diene polymers that absorb light and produce the characteristic deep brown-colored precipitates observed in solutions of oxidized dopamine. Similar reactions for its precursors tyrosine and L-DOPA are responsible for the formation of melanins and related products which serve as biological pigments and account for such biological phenomena as the browning of bananas and the tanning of skin and cuticles [7, 16, 17]. In vitro studies suggest that the generation of a reactive oxygen species, which can then lead to covalent modification of thiol-containing proteins by oxidized DA,

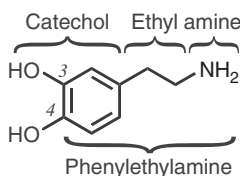


Figure 7.1 Chemical structure of DA. The various named moieties are delimited by the brackets. The numbering of positions around the phenyl ring is indicated.

is of potential concern for Parkinson's patients taking chronic, high doses of L-DOPA and who are in a perpetual state of neurodegeneration-induced secondary oxidative stress as this has been proposed to exacerbate degeneration [18, 19]. However, this has recently been shown not to occur to a significant extent in vivo [20, 21]. Nevertheless, the formation of certain in vitro neurotoxic dopamine metabolites, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL), salsolinol, or *N*-methyl(*R*)-salsolinol [22–24], may still be a source of concern [25].

Aside from its role as a precursor of pigments, in animals, DA also serves as a signaling molecule. Dopamine is considered a neurotransmitter when it is released from neuronal synapses and acts locally on presynaptic or postsynaptic DA receptors or a neurohormone when its release is humoral, meaning that it is transported to a distant DA receptor site through blood circulation. Although both synaptic and humoral responses to DA are observed in the periphery and the CNS, within the CNS DA is predominantly a neurotransmitter. An example of a humoral response in the CNS is the tonic, pulsatile release of DA from the arcuate nucleus of the hypothalamus that regulates prolactin secretion from the anterior pituitary. A humoral-type response in the periphery is the nausea induced by high plasma levels of peripheral DA via stimulation of DA receptors in the chemoreceptor trigger zone. In order to reduce such a side effect, L-DOPA is coadministered with peripheral inhibitors of the enzyme aromatic amino acid decarboxylase (AADC), such as carbidopa, which prevent the peripheral conversion of L-DOPA to DA.

Dopamine is a derivative of the aromatic amino acids phenylalanine and tyrosine and a precursor of the neurotransmitters norepinephrine and epinephrine. The anabolic and catabolic pathways for DA, as well as agents that block the enzymes that perform these conversions, are shown in Figure 7.2. Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of DA, and consequently, in situ intracellular localization of this enzyme is a popular marker for dopaminergic (and noradrenergic) cells. Due to its key role in DA anabolism, tyrosine hydroxylase is under heavy regulation at both the transcriptional and posttranslational levels [26–28]. Only one protein isoform of tyrosine hydroxylase is present in rats, while, due to differential splicing, two are present in nonhuman primates and four in humans [29, 30]. L-DOPA is converted to dopamine by AADCs associated with biogenic amine-containing neurons [31]. The ability of striatal serotonergic neurons and glia to convert L-DOPA to dopamine may explain why L-DOPA continues to be effective, albeit to a lesser extent, in advanced stages of Parkinson's disease when fewer dopaminergic neurons are remaining in the substantia nigra [32–34]. Once formed, DA can be converted by the enzyme dopamine- β -hydroxylase, which is present in noradrenergic neurons, or it can undergo catabolism or be taken back up following release. The two most reliable measures of DA turnover are 3,4-dihydroxyphenyl acetic acid (DOPAC)–DA and homovanillic acid (HVA)–DA ratios. HVA–DA ratios in cerebrospinal fluid (CSF), in particular, serve as an accurate measure of brain dopaminergic activity. In contrast to phase I catabolism, there are notable species and cell-type differences in the phase II catabolism of DA. For example, in rats, the major excreted metabolites are glucuronidated, while in humans they are sulfonated [35–37]. Differences in phase II metabolism of DA are also observed in cell lines derived from different tissues [38].

Drugs that regulate dopaminergic metabolism play a central role in the therapeutic management of diseases and disorders. For example, inhibitors of the catabolic enzyme monoamine oxidase (MAO) B extend the actions of DA by slowing

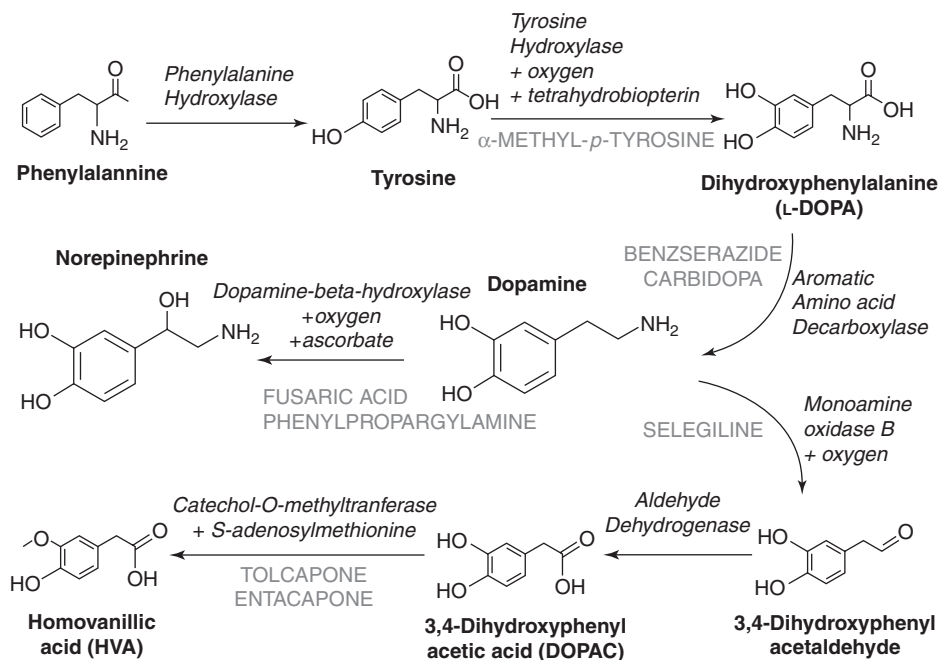


Figure 7.2 Metabolic pathways for DA. The names and abbreviations for the various chemical species are indicated in bold face. The enzymes responsible for the different conversion steps are indicated in italic and their corresponding inhibitors are indicated in capital text.

its degradation and therefore are sometimes used as treatments for disorders characterized by an direct or indirect hypodopaminergic state, like Parkinson's disease and ADHD, respectively. MAO_B-selective inhibitors like selegiline are effective in preventing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity, because they block the enzymatic conversion of protoxin MPTP to its toxic metabolite 1-methyl-4-phenylpyridine (MPP⁺) [39]. Although the distinctions are not absolute, MAO_B is generally associated with the metabolism of DA and tyramine, while MAO_A is primarily responsible for norepinephrine and serotonin metabolism. Older generation inhibitors of MAO carry a risk for pressor amine-induced hypertensive crisis or, when taken in conjunction with inhibitors of serotonin reuptake, serotonin syndrome, because many are irreversible inhibitors and most are only sparingly selective for the MAO_B versus the MAO_A subtypes. New MAO inhibitors that are reversible and highly selective for the A or B isoforms are becoming available (e.g., moclobemide and lazabemide, respectively) and should prove to be much safer alternatives. Catechol-*O*-methyl transferase (COMT) inhibitors are commonly coadministered to those receiving L-DOPA to counter the observed upregulation of this enzyme that results from chronic treatment. This prevents the peripheral conversion of L-DOPA to its 3-methoxy derivative that competes for active transport but that cannot then be converted to DA once inside the brain.

7.3 HISTORICAL CLASSIFICATION AND MOLECULAR PROPERTIES OF DA RECEPTORS

Dopamine is known to exert its actions through the binding and activation of specific cell surface receptors which are members of the G-protein-coupled receptor (GPCR) super gene family. Before it was established that multiple subtypes of DA receptors exist, the stimulatory effect of dopamine on adenylyl cyclase activity in the neostriatum was demonstrated [40]. Subsequent testing of dopaminergic agonists revealed that some promoted inhibition rather than stimulation of adenylyl cyclase in the striatum [41]. This discrepancy led to the classification of D₁ receptors as those which stimulate adenylyl cyclase activity and raise intracellular levels of cyclic adenosine monophosphate (cAMP) and D₂ receptors as those which inhibit adenylyl cyclase activity (reviewed in [42]). In addition to regulating adenylyl cyclase, many other intracellular effects have now been ascribed to DA receptor stimulation ([43]; and see also Section 7.4).

Through the application of molecular biological techniques, we now know that there are D₁ and D₂ *subfamilies* of receptors rather than singular receptor subtypes. The D₁ DA receptor subfamily includes the “classical” D₁ (also initially called the D_{1A}) receptor, as well as the D₅ (also initially called the D_{1B}) receptor. The D₅ receptor differs from the D₁ receptor in primary amino acid sequence and anatomical distribution but binds D₁ receptor-selective agonists and antagonists with similar affinity. The D₂ receptor subfamily includes the classical D₂ receptor, which is found in a short and long isoform (D_{2S} and D_{2L}), and also the D₃ and D₄ receptors. These D₂-like receptors exhibit a variety of pharmacological, structural, and in some cases functional similarities.

7.3.1 The D₁ Receptor Subfamily

Early molecular cloning work led to the discovery that there are actually two distinct DA receptor subtypes which are coupled to the stimulation of adenylyl cyclase. The first of these, termed the D₁ receptor, was independently cloned by four separate groups using either the polymerase chain reaction with degenerate primers derived from previously cloned GPCR sequences or homology screening with a D₂ receptor probe [44–47]. These methods enabled the isolation of complementary deoxyribonucleic acids (cDNAs) or genes from either human or rat DNA libraries. Both the rat and human D₁ receptor genes encode a 446-amino-acid protein (see Fig. 7.3). Overall, the rat and human receptors are 91% homologous at the amino acid level.

The structure of the D₁ receptor deduced by hydropathy analysis is consistent with that of other GPCRs, with seven hydrophobic domains predicted to traverse the plasma membrane. The amino acids in these domains are thought to be in the α -helical configuration. Predicted asparagine-linked glycosylation sites are found on the amino terminal domain and the second extracellular loop. The D₁ receptor has a relatively small third cytoplasmic loop, similar to other biogenic amine receptors which are coupled to the stimulatory guanyl nucleotide binding protein Gs [48, 49]. In the third cytoplasmic loop are found several serine and threonine residues as well as a consensus recognition sequence for the cAMP-dependent protein kinase, which are potential sites of phosphorylation. Other sites of postranslational modification include a cysteine residue in the carboxyl terminal domain, which is palmitoylated,

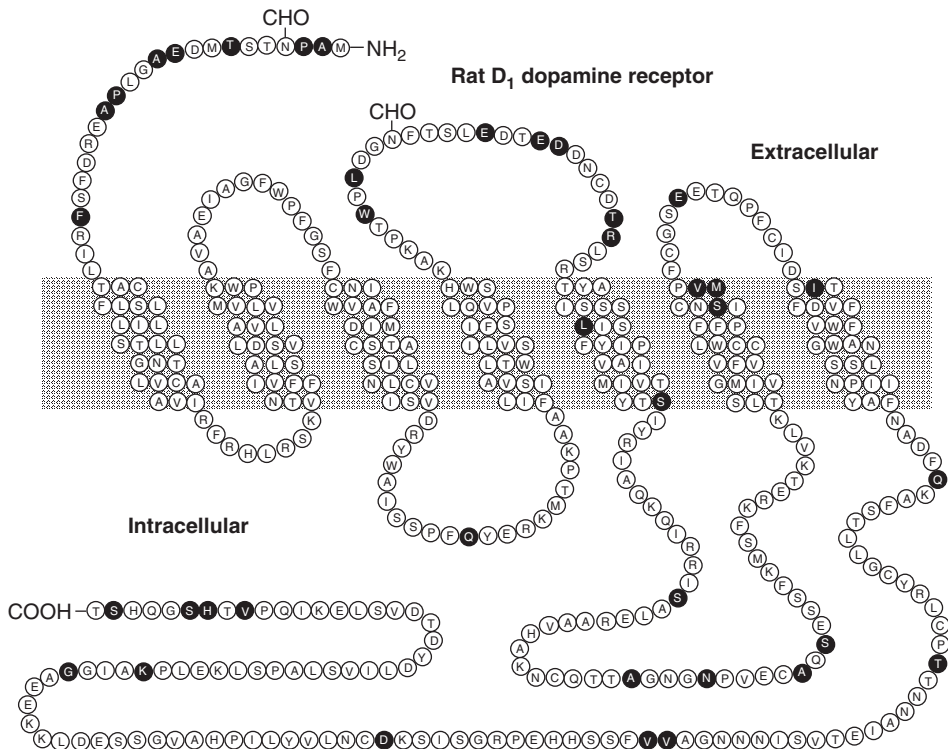


Figure 7.3 Membrane topography of rat D₁ DA receptor. Membrane-spanning domains are defined on the basis of hydropathy analysis. CHO: *N*-linked glycosylation sites. Solid residues indicate differences with the human receptor sequence.

and numerous serine and threonine residues also in the carboxyl terminus which serve as sites of G-protein-coupled receptor kinase (GRK)-mediated phosphorylation [50].

The human D₁ receptor gene has been mapped to chromosome 5 [46], and the 5' flanking regions of the rat and human D₁ genes have been studied [51–53]. While the coding region is intronless, the rat and human D₁ receptor genes contain a small intron in the 5' noncoding region. The promoter does not contain a TATA box or CAAT box; however, several SP1, AP1, and AP2 sequences have been identified. Also found in the promoter region is a cAMP responsive element recognition sequence.

Localization of the D₁ receptor messenger ribonucleic acid (mRNA) expression has been mapped using Northern analysis and in situ hybridization (for a review, see [54]). Expression of D₁ receptor mRNA is highest in the caudate putamen, nucleus accumbens, and olfactory tubercle. Lower levels of expression are found in the basolateral amygdala, cerebral cortex, septum, thalamus, and hypothalamus. D₁ receptor binding activity measured with the antagonist [³H]SCH-23990 is closely matched with mRNA expression in the striatum, amygdala, nucleus accumbens, and suprachiasmatic nucleus, consistent with the belief that D₁ receptors are localized largely on neuronal cell bodies rather than nerve terminals in these nuclei [55].

The second D₁-like receptor, referred to as the D₅ receptor, was isolated from human genomic libraries in 1991 [56–58]. The human D₅ receptor, which contains 477 residues, shares 50 and 80% homology with the human D₁ receptor in their coding regions and transmembrane-spanning domains, respectively. The rat homolog of this receptor was initially termed the D_{1B} receptor, consistent with classification of the receptor in the D₁-like subfamily [59, 60]. The rat D_{1B} receptor encodes a 475-amino-acid protein which is 83% homologous with its human homolog, the D₅ receptor. Like the D₁ receptor, the D_{1B} and D₅ receptors have asparagine-linked glycosylation sites in the amino terminus and the second extracellular loop, a small third cytoplasmic loop with putative phosphorylation sites, and a cysteine residue in the carboxyl terminus. The coding region in these genes also lacks introns [56–58] as in the D₁ receptor gene.

In the human genome, two pseudogenes related to the D₅ receptor have been isolated and termed *D5ψ1* and *D5ψ2* [56, 58, 61]. The nucleotide sequences of these pseudogenes are 95% conserved with the human D₅ sequence. Expression of functional proteins from the sequences does not occur due to insertions and deletions which encode termination codons. The *D5ψ1* gene has been shown to be transcriptionally active, and the resulting *D5ψ1* mRNA is recognized by most probes used for in situ hybridization analysis of the D₅ receptor [62]. The *D5ψ2* appears to be transcriptionally active as well [63]. In the human, the D₅ receptor gene and the pseudogenes are not found on the same locus, as the D₅ receptor gene has been mapped to chromosome 4 and the *D5ψ1* and *D5ψ2* to chromosomes 1 and 2, respectively [61, 64, 65].

Both the rat and human D₅ receptor homologs stimulate adenylyl cyclase activity and bind D₁-selective ligands with similar affinities as the D₁ receptor. While no ligands exhibit substantial preference for either subtype, DA binds with a 5- to 10-fold higher affinity to the D₅ receptor than to the D₁ receptor [56–60]. The reason for this high degree of pharmacological similarity is probably due to high structural homology that these receptors show in the transmembrane-spanning/ligand binding domains. It remains a formidable task for medicinal chemists to design drugs that are significantly selective for either of these D₁-like receptors. Another notable distinction between the two D₁-like receptors is that the D₅ receptor has been suggested to be constitutively active when expressed in HEK-293 cells [66].

The localization pattern of D₅ receptor mRNA differs significantly from that of the D₁ receptor. Interestingly, D₅ receptor mRNA expression has not been observed in the striatum using Northern or in situ hybridization analysis [57, 58, 60], suggesting that this receptor may not play as large a role in motor control as the D₁ receptor. In the rat, regions where D₅ receptor mRNA expression is observed to be high include the olfactory tubercles, hippocampus, hypothalamus, and mamillary bodies. Localization of D₅ receptor binding by in vitro autoradiography is currently not possible due to the absence of a highly selective D₅ receptor radioligand.

7.3.2 The D₂ Receptor Subfamily

As the D₂ receptor was the first DA receptor to be cloned, its isolation was dependent on homology to a similar GPCR. Low-stringency screening of a rat genomic library with a β₂-adrenergic receptor probe yielded homologous DNA fragments which were

subsequently used to isolate cDNA clones from a rat brain library. One of these clones was found to be the D₂ receptor [67]. The human D₂ receptor homolog was subsequently cloned and found to be 96% identical with the rat receptor with one amino acid deletion [68–71]. The human D₂ receptor gene has been mapped to chromosome 11 [72]. Structurally, the D₂ receptor contains a large third cytoplasmic loop, a short carboxyl terminal tail and three asparagine-linked glycosylation sites in the amino terminal region (see Fig. 7.4). The main structural difference between the D₂ and the D₁ receptor subfamilies is that the D₂-like receptors have large third cytoplasmic loops and short carboxyl termini, structural motifs that are characteristic of G_{i/o}-coupled receptors.

When transfected into mammalian cells, cloned D₂ receptors have been shown to activate a variety of signal transduction pathways ([43]; see also Section 7.4). In addition to adenylyl cyclase inhibition [73], these include stimulation of arachidonic acid release [74, 75], phosphatidylinositol hydrolysis and mobilization of calcium [76], regulation of K⁺ channels [77–79], and suppression of prolactin release [80]. With a few exceptions, these effects are similar to those observed in endogenous D₂ receptor-expressing tissues. The signaling properties of D₂-like receptors are discussed in detail below.

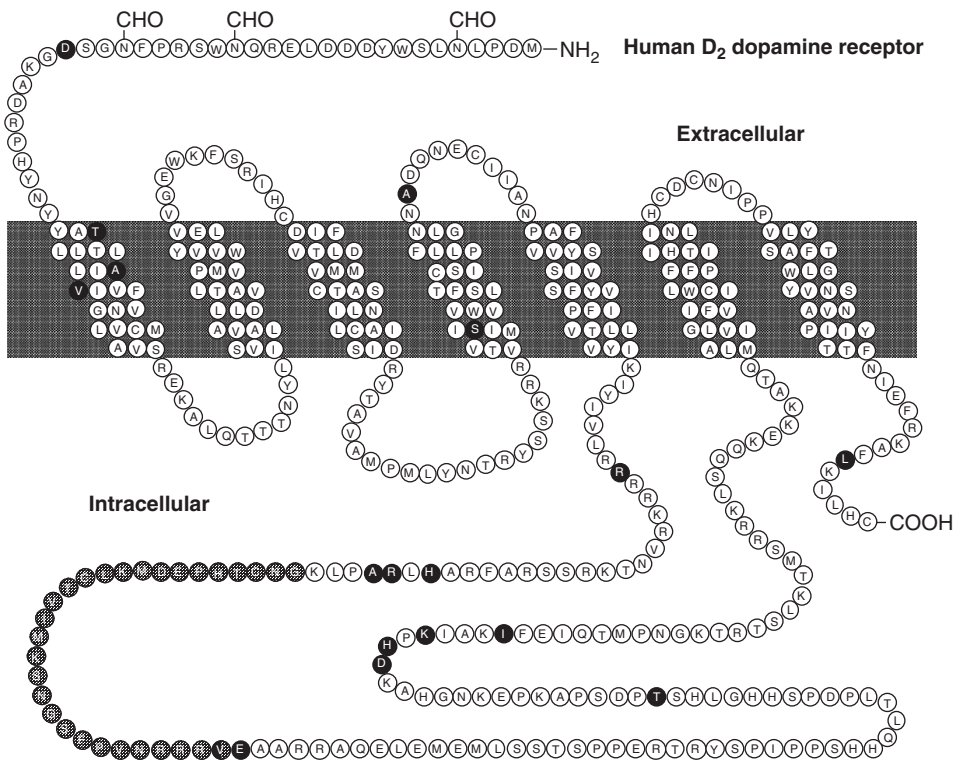


Figure 7.4 Membrane topography of human D₂ DA receptor. Membrane-spanning regions are defined on the basis of hydrophathy analysis. The hatched circles indicate the alternatively spliced exon of the D₂ receptor. The solid circles indicate differences with the rat receptor sequence. CHO: N-linked glycosylation sites.

Many studies have been conducted to investigate the localization of D₂ receptor mRNA in the brain [81–86]. The D₂ receptor mRNA distribution correlates well with previous information on D₂ receptor distribution generated using receptor binding and autoradiography methods. The areas of highest expression in the brain include the caudate putamen, nucleus accumbens, and olfactory tubercle. Receptor mRNA is also found in dopaminergic cell bodies within the substantia nigra pars compacta and ventral tegmental areas, suggesting an additional presynaptic role for the D₂ receptor. Cellular localization of the D₂ receptor mRNA has also been investigated in the striatum, where about 50–75% of the medium-sized cells appear to express receptor mRNA [87–89]. Recent evidence indicates that most of the cells which express the D₂ receptor mRNA in high abundance are enkephalinergic neurons [54, 87]. D₂ receptor mRNA has also been observed in large-diameter cells in the striatum, the majority of which appear to be cholinergic interneurons [54].

The D₂ receptor RNA is known to undergo alternative splicing, as two isoforms with different-sized coding regions have been isolated [59, 68–70, 90–92]. The D₂ long (D_{2L}) receptor contains a 29-amino-acid sequence which is absent from the D₂ short (D_{2S}) receptor. This sequence is encoded by one of the eight exons of the D₂ receptor gene [68, 70, 72, 93–95]. Both isoforms are found in human, rat, bovine, and murine tissue. They share similar pharmacological profiles and functional attributes, although they may act via different G proteins to mediate adenylyl cyclase inhibition [68, 96] and to regulate potassium channels [77–79]. The function of the 29-amino-acid sequence may also be to target the receptor isoforms to different regulatory pathways. This hypothesis stems from observations of differential effects of agonist pretreatment on the different receptor isoforms. Zhang *et al.* [97] found that agonist pretreatment of D_{2L} receptor-expressing cells resulted in upregulation of receptor expression whereas a similar treatment of D_{2S} receptor-expressing cells resulted in receptor downregulation.

The second receptor in the D₂-like subfamily to be identified was the D₃ receptor. Cloned in 1990 [98], the D₃ receptor shares 52% overall homology and 75% transmembrane homology with the D₂ receptor. The rat D₃ receptor is 446 amino acids long and contains asparagine-linked glycosylation sites in the amino terminus, a cAMP-dependent protein kinase recognition sequence in the third cytoplasmic loop, and a cysteine residue in the carboxyl terminal domain. The human D₃ receptor gene, which encodes a 400-amino-acid protein, has been cloned and mapped to chromosome 3 [99, 100]. The pharmacological profile of the D₃ receptor is similar to that of the D₂ receptor [98, 101–103].

The D₃ receptor is the only cloned DA receptor which is guanine nucleotide insensitive and relatively ineffective in regulating adenylyl cyclase activity (however, see below). However, regulation of DA release [104, 105], stimulation of neurite extension and branching [106], and activation of *c-fos* and mitogenesis [107] have all been identified as D₃ receptor-mediated events. These effects may be mediated by a second-messenger system that has yet to be elucidated. Seabrook *et al.* [108] have also shown that the D₃ receptor can depress Ca²⁺ currents in transfected NG108-15 cells. Similarly, Liu *et al.* [79], also working with NG108-15 cells, have found that the D₃ receptor can directly couple to the modulation of K⁺ currents.

In brain, D₃ receptor mRNA localization is greater in hypothalamic and limbic nuclei such as the olfactory tubercle, islands of Calleja, hippocampus, nucleus accumbens, and bed nucleus of the stria terminalis than in the basal ganglia [54,

109]. Minimal expression is observed in the caudate and putamen. D₃ receptor protein has been difficult to investigate due to the limited ligand specificity between D₂ and D₃ receptors. Use of [¹²⁵I]iodosulpiride in the presence of domperidone to reduce D₂ receptor labeling by autoradiography reveals the highest levels of D₃ binding in the islands of Calleja and the nucleus accumbens [110].

The D₃ receptor gene contains several introns and alternative splicing has been observed to produce nonfunctional variants [111, 112]. A functional variant has been identified in mice which lacks a 63-bp sequence in the third cytoplasmic loop [113]; also see Section 7.6).

The last D₂-like receptor to be identified was the D₄ receptor. Cloning and expression of this receptor was accomplished in several stages [114]. A partial-length human D₄ receptor cDNA was isolated by homology screening of a neuroblastoma cell library with a D₂ receptor probe. With this fragment used as a probe, a clone was isolated from a human genomic library which contained the coding sequence of the D₄ receptor. A full-length, correctly spliced D₄ receptor cDNA clone was subsequently isolated from a library constructed from COS cells which had been transfected with the D₄ receptor genomic DNA. The human D₄ receptor is 387 amino acids long (however, see below) and shares 41 and 56% homology with the D₂ receptor coding and transmembrane regions, respectively. Its large third cytoplasmic loop contains a cAMP-dependent protein kinase recognition site and its amino terminus contains one asparagine-linked glycosylation site.

The gene encoding the D₄ receptor, located on chromosome 11 [115], has been shown to be polymorphic [116]. A 48-bp sequence encoding 16 amino acids in the third cytoplasmic loop is repeated from two to nine times, with four repeats occurring most frequently in the population. The pharmacological profiles of D₄ receptors with varying repeat lengths appear to be similar [117]. The potential significance of this polymorphism is discussed in Section 7.6.

The binding properties of the D₄ receptor closely resemble those of the D₂ receptor [105, 114, 117–119]. As with D₂ receptors, agonist binding to D₄ receptor is guanine nucleotide sensitive. D₄ receptors are coupled to inhibition of adenylyl cyclase when transfected into a variety of mammalian cell lines [119]. In the MES23.5 neuroblastoma cell line, D₄ receptors have been shown to reduce potassium influx [120].

In rodents, the highest expression of D₄ receptor mRNA is found in the heart [118]. In rodent brain, expression is about 10-fold lower than in heart, with the highest levels observed in the frontal cortex, amygdala, olfactory bulb, and hypothalamus [114]. Very low levels of expression are detected in the olfactory tubercles and striatum.

7.4 DA RECEPTORS AS SIGNAL TRANSDUCERS

Dopamine transmits signals across cellular membranes by interacting with membrane-bound signal-transducing receptors that induce changes in the level of intracellular second messengers. Like other rhodopsin like class A GPCRs, DA receptors couple to intracellular guanosine triphosphate (GTP)-sensitive heterotrimeric G-protein complexes, although D₃ receptors may be an exception to this (see Section 7.5). The G-protein subtype coupling preference of the receptor determines

TABLE 7.1 Typical G_α Protein Coupling Preferences for Dopamine Receptors

Subfamily	Subtype	G_s	$G_i/G_o/G_z$	G_q	G_t	Comments
D ₁ -like	D ₁	+				↑ cAMP
	D ₅	+				↑ cAMP
D ₂ -like	D ₂		+			↓ cAMP
				+		↑ IP ₃
	D ₃		+			↑ Mitogenesis
			+			↑ Mitogenesis
			+		+	↓ cAMP
	D ₄		+			↑ Mitogenesis

Abbreviations: cAMP = cyclic adenosine monophosphate, IP₃ = 1,4,5-inositol triphosphate.

^aThe subscript designations indicate the G_α protein subfamily.

the type of intracellular second-messenger response. This selectivity of G-protein coupling is controlled primarily by the subtype of the α subunit (Table 7.1) and is secondarily modulated by the subtype of β and γ subunits. However, promiscuous coupling can occur when the levels of expressed G protein or receptor are very high, which suggests that the coupling preferences are by no means absolute. In general, though, the levels of receptors under physiological conditions are low enough to permit selective coupling by the different receptor subtypes. However, it is becoming apparent that heterooligomerization of DA receptors with other non-DA GPCRs can dramatically affect G-protein coupling preferences. For instance, D₂ receptors are known to prefer to couple with $G_{i/o}$ over G_s , but when coexpressed with cannabinoid CB1 receptors, this D₂ receptor-mediated coupling preference is reversed [121]. Although the strength of coupling to G-protein subfamilies and subtypes (isoforms) is known to vary among the DA receptor subtypes, the extent of intracellular signaling can depend in large part upon the presence of the appropriate isoforms of the enzymes activated by the G proteins. For example, D₃ receptor stimulation only leads to a robust increase in cAMP when the adenylyl cyclase isoform V is present [122]. The presence or absence of other receptors or specific enzyme isoforms has major implications for the development of tissue- or cell-specific drug responses.

Notwithstanding the above caveats, the canonical wisdom is that both of the D₁-like DA receptors prefer to couple to G_{α_s} proteins which stimulate the enzyme adenylyl cyclase, while all the D₂-like DA receptors couple to $G_{\alpha_{i/o}}$ proteins that inhibit this enzyme and the pertussis toxin-insensitive G_{α_z} [123]. D₂, D₃, and D₄ receptors have been shown to induce mitogenesis in serum-sensitive cell lines via a G-protein-dependent mechanism [107, 124–130]. D₂ receptors have also been shown to couple to G_{α_q} , and thereby stimulate the enzyme phospholipase C [131]. Remarkably, the D₄ receptor is among the growing number of known nonopsin receptors that couple to transducin (G_{α_t}) [132–134], which is in line with the localization of D₄ receptors in the retina [135, 136].

The GPCR cycle begins when an agonist binds to the receptor and converts it from the inactive low-affinity state to the active high-affinity state (Fig. 7.5). This induces a conformational change in the receptor that allows the inactive form of the heterotrimer G-protein complex to bind. This in turn promotes the exchange of guanosine diphosphate (GDP) for GTP from the α subunit of the G-protein in the presence of

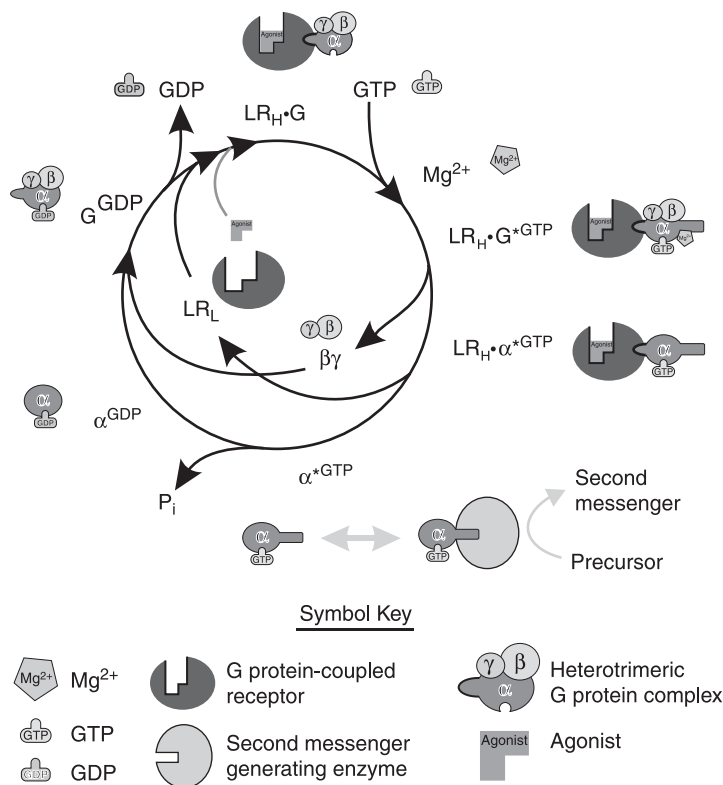


Figure 7.5 Schematic showing critical components of signaling cycle for heterotrimeric GPCRs. (See color insert.)

magnesium ions and activates the G-protein complex. The activated GTP-bound α subunit of the G-protein complex undergoes a conformational change, which promotes the dissociation of its G-protein β and γ subunits as well as its own dissociation from the receptor. Once the receptor is no longer bound by G protein, it converts back to the low-affinity state, which promotes the dissociation of agonist. In some cases the free β and γ subunits may associate with ion channels or other proteins and modulate their activity. The activated GTP-bound α subunit associates with effector enzymes and either potentiates or inhibits their activity until the bound GTP is autohydrolyzed to GDP by the G protein. This results in a change in the conformation of the inactivated GDP-bound α subunit, which favors dissociation from the enzyme and reassociation with its β and γ subunits, and the cycle begins anew.

7.5 MOLECULAR PHARMACOLOGY OF DA RECEPTORS: STRUCTURE–AFFINITY AND STRUCTURE–ACTIVITY RELATIONSHIPS

Structure–affinity and structure–activity relationship studies coupled with molecular modeling have revealed that three major physicochemical forces influence the interactions of DA with DA receptors (see review in [137]). The primary amine portion of

DA is protonated when bound to the binding site crevice, which allows it to form a high-energy reinforced ionic bond with a negatively charged and conserved aspartic acid (3.32, for an explanation of the universal naming system for the position of amino acids within GPCRs see [138]) present in the receptor [139, 140]. In general, the catechol hydroxyls of DA form hydrogen bonds to one of the conserved serines (S5.42) and, to a lesser extent, with one of the other two adjacent serines (S5.43 or S5.46) in the receptor. While none of these three serines are essential for D₁ or D₂ DA receptor activation by agonists [141–143], at least two (any two) serines are essential for the coupling of the D₂ receptor with G proteins and consequently essential for DA activation of the D₂ dopamine receptor [143]. Although β -phenylethylamine, which is a DA derivative lacking both hydroxyl groups, can still bind to both a high- and low-affinity state of the receptor [144] and by extension should be able to activate it, its affinity as well as the affinities for *meta*- and *para*-tryamine is greatly reduced [143, 145]. Thus, it appears that serines at positions 5.42, 5.43, and 5.46 serve to increase the binding affinity of DA and to anchor it in the binding site crevice, which would orient its aromatic ring such that it sterically clashes with a conserved residue in the transmembrane TM6 aromatic cluster (possibly 6.51 or 6.52). By analogy with other GPCRs, such a clash is believed to result in conformational changes in the receptor that are ultimately responsible for its activation [146]. Accordingly, many DA agonists have structural features similar to DA and interact with similar microdomains. An exception to this is quinpirole, which apparently does not interact with the conserved serine microdomain in TM5 of D₂ or D₃ receptors [142, 145, 147, 148]. A few rigid, fused heterocyclic agonists are subfamily selective. For example, SKF38393 and dihydrexidine are D₁-like selective, and (–)-quinpirole, apomorphine, propylnorapomorphine, 2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene (5,6-ADTN), 6,7-ADTN, and 7-hydroxy-2-(*N,N*-di-*n*-propylamino)tetralin (7-OH-DPAT) are D₂-like selective. Dopamine receptor antagonists (and inverse agonists) have more divergent structures but still invariably incorporate a protonatable amine pharmacophore (see, e.g., [10]). The binding of some inverse agonists and antagonists, like butyrophenones and substituted benzamides, appears to depend upon conserved aromatics in TM6 (6.51 or 6.52) of the receptor [149, 150]). However, the manner in which antagonists and inverse agonists interact with the aromatic residue at position 6.52 must differ from the agonists, because antagonists do not activate the receptor. By definition, allosteric modulators of DA receptors interact at a site distinct from the orthosteric site, that is the site where DA binds (for a recent exhaustive review of allosteric modulators of DA receptors see [151]).

Although one or more of the three conserved microdomains are important for ligand binding, they cannot fully account for the binding of receptor subtype-selective ligands, because, as expected, selective ligands tend to take advantage of additional nonconserved receptor subtype-specific amino acids. For example, most 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) with extreme selectivity for the D₄ DA receptor interact favorably with an aromatic microdomain that spans the interface of the second and third transmembrane (TM2/TM3) domains of the D₄ receptor [152, 153]. Other DA receptors, such as the D₂ receptor, also have aromatic residues in the TM2/TM3 microdomain, but their placement within this microdomain is different from that of the D₄ receptor (V2.61 and F3.28 in D₂ and F2.61 and L3.28 in D₄). This may explain why the majority of D₄-selective drugs are 1,4-DAPs with related substructural features [154]; however, it does not explain why

they have a range of functional properties [152]. A couple of highly D_4 -selective 1,4-DAPs (PNU101,387G and R010-4548), with uniquely positioned electronegative substructures, have moderate interactions with only one portion of this TM2/TM3 microdomain and thus must take advantage of additional unique microdomains on the D_4 receptor or their unique structural features are differentially exploited among the receptor subtypes [152]. Furthermore, D_2 -selective 1,4-DAPs, such as methylspiperone and aripiprazole, possess unique substructural features of their own and do not have major interactions with any portion of the TM2/TM3 microdomain [153, 155]. A conserved aromatic microdomain in TM6 (residues F6.51, F6.52, and H6.55) has been implicated in the high-affinity binding of substituted benzamides, like sulpride, or butyrophenones, like methylspiperone [150], although it is unclear whether in the case of residues F6.51 and F6.52 this is a direct effect on ligand binding or a more global effect on protein structure. How molecular recognition is achieved for other much less subtype-selective chemical classes of atypical antipsychotics, including the benzapines (e.g., clozapine, olanzapine, quetiapine, loxapine) and benzazolines (e.g., risperidone, ziprasidone), remains to be elucidated [137].

7.6 SPLICE VARIANTS, SNPS, AND OTHER POLYMORPHISMS WITH FUNCTIONAL CONSEQUENCES

As discussed above, five genotypically distinct DA receptors have been cloned from humans and the corresponding homologs are present in monkeys, rats, and mice. To date alternative splice variants have been identified for D_2 and D_3 DA receptors. Both a short (D_{2S}) isoform and a long (D_{2L}) isoform, which contain a 29-amino-acid insertion in the third intracellular loop (ICL3), have been identified in humans, rats, and mice (see Section 7.3), although in mice the long isoform predominates [94]. In *Caenorhabditis elegans* both a long and a short ICL3 splice variant of a D_2 DA receptor ortholog have been identified [156], while in *Drosophila* eight D_2 receptor orthologs have been identified and each is due to alternative splicing in ICL3 [157]. However, such alternative splicing apparently does not occur in *Xenopus*, rather two distinct D_2 genes are present [158].

Although there are no large differences in the affinity for DA or other dopaminergic drugs for the D_{2S} and D_{2L} variants, only the D_{2L} isoform couples to $G\alpha_{i2}$ [159]. Furthermore, D_{2L} , but not D_{2S} , interacts with heart-type fatty acid binding protein [160] and activates NF-kappaB in a calcineurin-dependent manner [161]. The presence or absence of the 29-amino-acid insert in the D_2 receptor is also important for trafficking of the D_2 receptor, because the D_{2S} isoform resides predominantly in the plasma membrane while the D_{2L} receptor is primarily retained in the endoplasmic reticulum [162] or resides in a perinuclear location around Golgi [160]. In addition, the D_{2S} receptor has a somatodendritic or presynaptic localization where it serves as an autoreceptor, while the D_{2L} receptor has a postsynaptic localization [163, 164]. Recently, a third isoform that is two amino acids longer than the D_{2L} isoform has been identified in humans, and remarkably its affinity is reported to be considerably higher than the other D_2 splice variants [165]. Both ethanol and estradiol modulate alternative splicing of the D_2 receptor in primary pituitary cultures, which results in a relative increase of the levels of D_{2L} with a concomitant decrease in the levels of D_{2S} [166, 167].

Similar to the D₂ DA receptor, a splice variant of the mouse D₃ DA receptor has been reported that results in a shortened ICL3 (21 amino acids less) [168]. In contrast, a splice variant of the D₃ receptor has also been identified in rat brain, which results in a 28-amino-acid elongation of the first intracellular loop (ICL1) [169]. A variety of splice variants have been reported that result in an array of nonfunctional truncated D₃ receptors that encompass transmembrane-spanning domains II, III (D₃ *TM4-del*), or all seven (D₃*nf*) but lack a carboxy terminus tail [112, 170, 171]. The *D3nf* variant alone has no demonstrable functional properties and does not bind dopaminergic ligands. However, *D3nf* reduces apparent receptor density (B_{\max}), as assessed by radioligand binding, when it is coexpressed with the full-length D₃ receptor [172] and it prevents trafficking of the full-length D₃ receptor to the plasma membrane [173]. This may have physiological consequences as *D3nf* is expressed in human brain [171] and it is upregulated in the brains of chronic schizophrenics [174]. Recently, it has been shown that the functional properties and the binding of ligands to the *D3nf* truncation variant can be rescued by coexpression of the carboxy terminal tail of the D₃ receptor [175].

Many single-nucleotide polymorphisms (SNPs) have been reported in both coding and noncoding regions of DA receptors, which is not surprising given that a survey of SNPs in a panel of eight different human GPCRs reveals a frequency of one polymorphism per 584 bp [176] and that DA receptors average ~ 1300 bp in length. Despite the expected high frequency of SNPs in DA receptors, thus far only one has been reported that results in a change in the coding sequence that leads to dramatic functional consequences. Specifically, a case study of a genetic variant of the human D₄ DA receptor has revealed that mutation of a conserved valine in TMS5 (D4.4-V5.40G) results in a receptor that is unable to be activated by DA, and that has drastically decreased affinities for DA, clozapine, and olanzapine, despite having comparatively smaller or no significant decrease in affinities for haloperidol and spiperone, chlorpromazine, and raclopride, respectively [177].

Over two dozen large polymorphic variants of the human dopamine D₄ receptor gene have been identified containing anywhere from 2 to 10 copies of a variable 16-amino-acid repeat unit in the third intracellular loop [116, 178, 179]. Tandem repeats in this same region (exon III) of the D₄ gene have been identified in a large number of mammals but appear to be absent from rodents [180]. The seven-repeat variant in humans has been reported to be associated with ADHD [181–185]. The original report, [181] elaborated upon the link between the seven-repeat polymorphism and ADHD based on three lines of evidence: the role of (hypo)dopaminergic transmission in ADHD, the distribution of D₄ receptors in the brain (e.g., prefrontal cortex and striatum), and the high polymorphic variability of the D₄ receptor gene that was suggested to have functional significance. Specifically, it was proposed that the seven-repeat form of the DA D₄ receptor is hypo responsive to DA compared to the other polymorphic variants. However, an in vitro study demonstrated only a very small difference in functional coupling to G proteins (two-fold decrease in potency and no change in efficacy) for the seven-repeat polymorphism (D4.7) relative to other numbers of repeats of this polymorphism [186]. Further, this small functional difference observed in the original study has not been reproduced by other groups [187], and additional studies by the original group of repeat polymorphisms of various lengths (D4.0–D4.10) have revealed no significant differences in either pharmacology (affinity) or functional coupling efficiency (potency or efficacy) [188].

Although multiple genetic studies implicate the D₄ receptor in ADHD [185, 189–194], even if the seven-repeat polymorphism of the human DA D₄ receptor is somehow linked to ADHD, which remains controversial [192, 194–200], it appears that differences in G-protein coupling efficiency alone cannot account for the behavioral symptoms characteristic of the disorder.

7.7 CLASSIFICATION OF DOPAMINERGIC DRUGS ACCORDING TO TREATMENT CATEGORY

Since dopaminergic systems regulate a variety of cognitive and motor behaviors, drugs that target DA receptors, transporters, and metabolic enzymes are vital to the pharmacotherapeutic management of neurological and psychiatric conditions through the palliative relief of selected symptom modalities. It is possible for dopaminergic drugs to treat a plethora of clinical disorders, because relatively segregated dopaminergic neuronal pathways tend to selectively modify motor and cognitive functions and similar symptoms may be present in different disorders. While drugs are typically classified on the basis of their molecular mechanisms of action, such as agonist, partial agonist, inverse agonist, or antagonist, it is often useful to group them according to their clinical applications.

7.7.1 Parkinson's Disease

Dopamine receptor agonists provide relief to Parkinson's disease patients by replenishing the lost dopaminergic activity in the striatum that results from the death of DA-producing neurons projecting from the substantia nigra. The most common antiparkinsonian drugs are the DA precursor L-DOPA, apomorphine, pramipexole, ropinirole, and the ergolines bromocriptine and pergolide [201]. Pergolide and apomorphine are sparingly selective for D₂-like over D₁-like DA receptors, and pramipexole and ropinirole are moderately selective for D₃ over D₂ [202, 203]. Although stimulation of D₃ DA receptors may be important for treating Parkinson's disease [204, 205], pramipexole has other properties that would be beneficial for slowing the progression of neurodegeneration. For example, it is an effective antioxidant at high concentrations and can block the mitochondria transition pore at low concentrations [206, 207]. Since chronic stimulation of D₂ DA receptors is associated with potentially severe extrapyramidal side effects, like tardive dyskinesia, efforts have focused on developing D₁-selective agonists [208]. Although this strategy may lack the neuroendocrine side effects associated with stimulation of D₂ receptors, it appears that selective stimulation of D₁ receptors still has some dyskinetic side-effect liability, and there are other side effects unique to D₁ receptors as well [209]. The dilemma concerning the use of D₁-selective agonists as antiparkinsonian agents is not so much that most D₁-selective agonists are not highly selective over D₂ or that many have low efficacy. Rather it is more that most D₁-selective agonists have low orally bioavailability or they rapidly desensitize D₁ receptors, producing tolerance [208, 210, 211]. An exception to the latter is for the sparingly D₁-selective (approximately six-fold selective over D₂) agonist dinapsoline [211, 212], but it remains to be seen whether it has reduced side-effect liability in humans.

7.7.2 Schizophrenia

Historically, the treatment of psychosis has been closely correlated with the blockade of D₂ DA receptors [213, 214]. However, the D₂-selective partial agonist aripiprazole has been shown to not only be effective in treating psychosis but also be free of the extrapyramidal and neuroendocrine side effects common of typical antipsychotics (e.g., fluphenazine and haloperidol), which are due to excessive D₂ receptor blockade [215, 216]. Thus, the utility of aripiprazole appears to be that it reduces dopaminergic activity without completely blocking it. This is in line with the finding that many atypical antipsychotics have reduced extrapyramidal and neuroendocrine side-effect liability and, in general, have lower affinity for the D₂ receptors than typical antipsychotics [217–219]. Remarkably, aripiprazole also has a superior side-effect profile compared to the newer atypical antipsychotics (i.e., clozapine, olanzapine, quetiapine, risperidone, and ziprasidone) as it lacks the cardiovascular and blood dyscrasia liability and has a reduced obesity/diabetes liability.

7.7.3 Bipolar Mania, Autism, Alzheimer's Disease, and Tourette's Syndrome

In addition to their role in treating schizophrenia, some of the second-generation atypical antipsychotics are also used to treat psychosis and/or agitation and aggression of the Alzheimer's type (see [220, 221] for reviews) as well as a range of symptom modalities related to other psychiatric or neurological disorders. For example, risperidone has been shown in double-blind placebo-controlled studies to effectively treat autism spectrum disorders [222, 223]. Atypical antipsychotics have also proved effective in treating bipolar mania (see [224] for a review), which is interesting in light of the fact that intense mania and paranoid schizophrenia share many of the same clinical features [225–228]. While typical antipsychotics (e.g., pimozide and haloperidol) are approved for the treatment of the tic component of Gilles de la Tourette's syndrome, atypical antipsychotics appear to be promising alternatives (see [229, 230] for reviews).

7.7.4 Attention-Deficit Hyperactivity Disorder

The psychostimulants amphetamine and methylphenidate are used to treat ADHD. These psychostimulants are indirect dopaminergic (and norepinephrine) agonists, meaning that they increase levels of synaptic DA (and norepinephrine) by altering the activity of synaptic transporters and/or the activity and distribution of vesicular monoamine transporter-2 [231, 232]. Such agents are useful for the treatment, of ADHD, because this disorder is associated with a (noradrenergic and dopaminergic) hypofrontality that results in an attenuated drive of dopaminergic output from subcortical structures [233–236]. Animal studies suggest that selective DA D₄ antagonists may have potential as non-psychostimulant anti-ADHD drugs. Neonatal rodents injected intracisternally with 6-hydroxydopamine (and a norepinephrine transport blocker to spare noradrenergic neurons) develop a temporary juvenile hyperactive phenotype, which has been used as a model for hyperactivity in ADHD. In rats, this hyperactivity is reversed by some D₄-selective antagonists (i.e., by CP293,019, L745,870, U101,958, but not by S-18126) and exaggerated by a D₄-selective agonist (CP226,269) [237, 238]. In neonatal 6-hydroxydopamine-lesioned

wild-type but not D₄ knockout mice, both hyperactivity and reduced behavioral inhibition are evident in juveniles, and the hyperactivity is reversed by the D₄-selective antagonist PNU101,387G [239]. Although in wild-type mice the lesion-induced hyperactivity is reversed by amphetamine, the role of the D₄ receptor with respect to psychostimulant sensitivity is unclear. For example, unlesioned D₄ knockout mice, like their unlesioned non transgenic littermates, become hyperactive when challenged with methylphenidate or amphetamine, and, incidentally, D₄ knockout mice are supersensitive to the psychostimulants cocaine and methamphetamine [240]. Interestingly, unlesioned D₄ knockout mice are hypoactive in an open field, even though they have enhanced performance in the rotarod test [240]. However, D₄ knockout mice also have enhanced unconditioned fear responses [241], which might contribute to their hypoactivity in an open field.

7.7.5 Substance Use

Although DA receptors have long been pursued as targets for the potential treatment of substance use, this goal has yet to be fully realized and the findings in this field tend to be controversial. For example, systemically administered D₁-selective agonists (e.g., ABT-431) reduce cocaine-seeking behavior in rats [242, 243] and humans [208]; however, others studies indicate that the D₁-selective agonist ABT-431 attenuates the subjective effects of cocaine, including ratings of the “high” and “quality”, without affecting the number of self-administrations [244]. Paradoxically, D₁-selective antagonists have also been reported to reduce cocaine-seeking behavior by some groups [245–247] but not others [248]. Still other studies report that the D₁-like-selective antagonist SCH39166 attenuates the euphoric effects of cocaine. Although the potential for drug addiction treatment strategies involving the D₁ receptor remain unclear, an association between a D₁ receptor gene polymorphism and “sensation seeking” in alcoholic men has been reported [249].

Like D₁-selective compounds, the role of D₃-selective compounds in drug-seeking behavior is controversial. Many of the studies have focused on the D₃-selective ligand BD897. While there is some debate as to whether BD897 acts a partial agonist or an antagonist *in vivo* [250–252], in either case the effects would presumably still lead to a reduction in responsiveness relative to the endogenous full agonist dopamine. BD897 has been reported by some groups to inhibit cocaine-seeking behaviors and morphine-induced place preference in rats [253, 254] and cocaine self-administration in rhesus monkeys [250, 255]. However, others have reported that BD897 (and other D₃-selective ligands) do not inhibit cocaine-induced place preference or cocaine self-administration in rats [256, 257]. Both BD897 and the D₃-selective antagonist ST-198 disrupt nicotine-conditioned place preference [258]. In addition, the D₃-selective antagonist SB277011A has also been reported to prevent nicotine-triggered relapse of nicotine-seeking behavior [259] and to attenuate alcohol consumption in rats [260].

7.7.6 Other Uses

There is some, albeit limited, evidence that neuroleptics, in particular risperidone, may prevent self-mutilation in disorders such as autism and Lesch-Nyhan syndrome [261–264]. Antipsychotics (usually pimozide) are used to treat monosymptomatic

hypochondriacal psychoses of the dermatological type, in particular, psychosomatic delusions of parasitosis [265–269]. Agonists with high selectivity for the D₄ DA receptor have been shown recently to induce penile erection in male rats and they continue to be evaluated for possible use in erectile dysfunction [270]. This may explain the clinical observation that sublingual application of the agonist apomorphine induces penile erection [271–274]. Recent double-blind and placebo-controlled clinical trials with the moderately D₃-selective agonist ropinirole have demonstrated that it is effective in treating restless-leg syndrome [275, 276]. Dopamine receptor antagonists that do not cross the blood–brain-barrier, such as domperidone, are effective antiemetics as they block the D₂ receptors residing on the peripheral portion of the chemoreceptor trigger zone [277].

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8

SEROTONIN SYSTEMS

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8.1	Introduction	257
8.1.1	Early History of 5-HT	259
8.2	Anatomy and Physiology of 5-HT System	260
8.2.1	Anatomy of 5-HT System	260
8.2.2	Synthesis of 5-HT	260
8.2.3	Degradation and Reuptake of 5-HT	261
8.3	5-HT Receptors	262
8.3.1	5-HT Receptor Nomenclature	262
8.3.2	5-HT ₁ Receptor Family	263
8.3.2.1	5-HT _{1A} Receptors	263
8.3.2.2	5-HT _{1B} and 5-HT _{1D} Receptors	267
8.3.2.3	5-HT _{1E} Receptors	268
8.3.2.4	5-HT _{1F} Receptors	269
8.3.3	5-HT ₂ Receptor Family	269
8.3.3.1	5-HT _{2A} Receptors	270
8.3.3.2	5-HT _{2B} Receptors	271
8.3.3.3	5-HT _{2C} Receptors	271
8.3.4	5-HT ₃ Receptors	273
8.3.5	5-HT ₄ Receptors	273
8.3.6	5-HT ₅ Receptor Family	274
8.3.7	5-HT ₆ Receptors	275
8.3.8	5-HT ₇ Receptors	275
8.4	Future of 5-HT Research	276
	References	277

8.1 INTRODUCTION

Although serotonin (5-hydroxytryptamine; 5-HT; Fig. 8.1) was discovered more than 50 years ago, 5-HT research continues to provide fertile soil for researchers

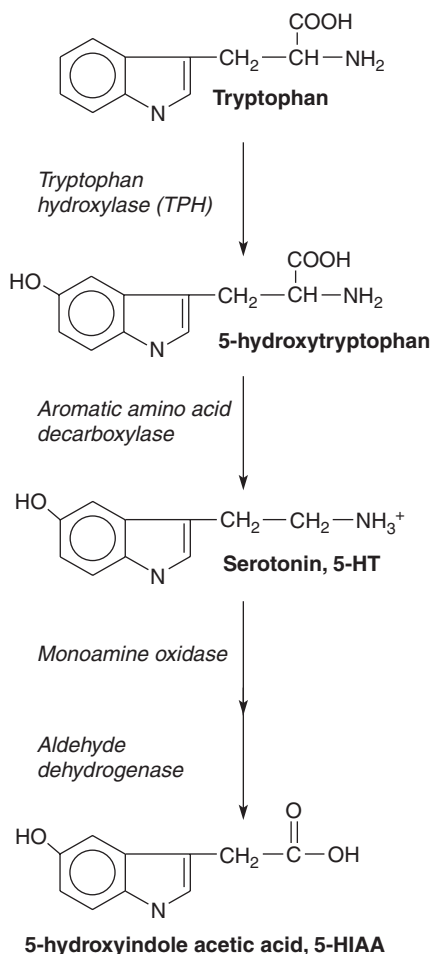


Figure 8.1 Biosynthesis and metabolism of 5-HT. The essential amino acid tryptophan is converted in a two-step pathway to 5-HT in serotonergic neurons originating in the brain stem raphe nuclei. The first enzyme in the pathway, tryptophan hydroxylase, is the rate-limiting step of 5-HT synthesis. 5-HT is degraded by the actions of monoamine oxidase (MAO) and aldehyde dehydrogenase into its primary metabolite, 5-hydroxyindole acetic acid (5-HIAA).

attempting to unravel the mysteries of central nervous system (CNS) functioning. It has only been in recent years, however, that the importance of 5-HT in the functioning of the human brain has begun to be understood. Thus, for instance, 5-HT has been implicated in the regulation of virtually all brain functions, including perception, mood, anxiety, pain, sleep, appetite, and aggression, as well as in the pathophysiology of many psychiatric and neurological disorders [1, 2]. The involvement of 5-HT in such an extensive range of functions implies that 5-HT may function as both a classical neurotransmitter and a neuromodulator. The neuromodulatory actions of 5-HT may provide an emotional or perceptual “tone” to transmitted information. Not surprisingly, 5-HT receptors and transporters continue to be a major focus of CNS drug discovery [2]. In fact, of the 10 top CNS drugs by total sales in the year 2003, 7 modulate 5-HT neurotransmission as part of their mechanism of

action (olanzapine, tradename Zyprexa; sertraline, Zoloft; venlafaxine, Effexor; risperidone, Risperdal; bupropion, Wellbutrin; paroxetine, Paxil; and quetiapine, Seroquel). The widespread use of serotonergic drugs has led to a growing public awareness regarding the central role 5-HT plays in regulating perception, mood, and emotion. Even with this wide armamentarium of 5-HT-modulating drugs, our understanding of the basic functions of 5-HT in the CNS remains limited. However, with the cloning of at least 15 different 5-HT receptors since the early 1990s and the subsequent development of receptor-subtype knockout mice, as well as development of compounds that are more highly selective at individual receptor subtypes, a greater understanding of 5-HT function continues to emerge. This knowledge will lead to newer, improved therapies with better efficacy or side-effect profiles and an enhanced understanding of neuropsychiatric disorders. This chapter will highlight the anatomy and physiology of the CNS 5-HT system with special emphasis on the subclasses of 5-HT receptors and give a brief overview of the importance of 5-HT in disease.

8.1.1 Early History of 5-HT

Serotonin was discovered in the late 1930s by Vittorio Erspamer at the University of Pavia in Italy while he was looking for substances that were capable of causing smooth muscle contraction. Such a substance was identified in enterochromaffin cells from rabbit gastric mucosa which Erspamer named “enteramine” [3, 4]. Subsequently, at the Cleveland Clinic in the late 1940s, the laboratory of Irving Page discovered and characterized a vasoconstricting substance in serum that was produced as soon as blood coagulated [5]. The vasoconstricting substance was subsequently crystallized and christened serotonin [6]; the structure of 5-HT was elucidated in 1949 [7]. While enteramine had an extensive place in the scientific literature during that time, it was not until 1952 that it was established that enteramine was identical to 5-HT [4].

In the 1950s, Betty Twarog joined the laboratory of Irving Page and hypothesized that invertebrate neurotransmitters might also be used as neurotransmitters in vertebrates and with her previous work (published two years later) showed that 5-HT was a neurotransmitter in mussels [8]. At that time, however, 5-HT was only a serum vasoconstrictor in mammals, and there was yet no reason to believe that it would have any function as a neurotransmitter. Her research in the Page laboratory resulted in the identification of 5-HT in the brain and thus issued the emergence of 5-HT in the field of neuroscience [9]. Despite the discovery of 5-HT in nervous tissue, there was at that time considerable resistance to the notion that 5-HT had any relevance for human brain functioning (E. Costa, personal communication) although some studies suggested a prominent role for 5-HT in modulating human consciousness [10]. The landmark studies by Brodie and colleagues which demonstrated that reserpine depleted 5-HT [11, 12] and that reserpine pretreatment interfered with some actions of chlorpromazine lent strong support to the hypothesis that 5-HT was a prominent modulator of CNS functioning. It was not until a few years later when Erminio Costa discovered 5-HT in the human brain [13] that psychiatric researchers began to concede a role for 5-HT in CNS functioning. Other pivotal studies in the early evolution of our notions regarding the role of 5-HT in CNS functioning came from seminal studies by George Aghajanian and colleagues [14] which demonstrated

that lysergic acid diethylamide (LSD) modulated 5-HT release in vivo and that the raphe neurons were the main source of brain 5-HT [15]. Studies by Steven Peroutka and Solomon Snyder demonstrated that [^3H]LSD labeled 5-HT receptors in the brain [16] and that multiple 5-HT receptors existed which had distinct functions [17].

8.2 ANATOMY AND PHYSIOLOGY OF 5-HT SYSTEM

8.2.1 Anatomy of 5-HT System

In the CNS, 5-HT is almost exclusively produced in neurons originating in the raphe nuclei that are located in the midline of the brain stem along its entire rostral-caudal axis. Since the first description of large cell bodies in the brain stem midline by Ramon y Cajal and a discerning of the anatomical architecture of the projections from these cell bodies [18–20], it became apparent that 5-HT-producing neurons form the largest and most complex efferent system in the human brain. The most caudal raphe innervate the medulla and the spinal cord, while the more rostral raphe, the dorsal raphe nucleus and the medial raphe nucleus, innervate much of the rest of the CNS by diffuse projections. The dorsal raphe nucleus projects thin 5-HT fibers that preferentially innervate the cerebral cortex, as well as the thalamus, caudate and putamen, nucleus accumbens, and dopaminergic nuclei of the midbrain (substantia nigra and ventral tegmental area). Conversely, the medial raphe nucleus projects thick 5-HT fibers with large varicosities that are relatively sparse but preferentially innervate the hippocampus and other limbic structures [21, 22]. The expansiveness of the serotonergic system with virtually every cell in the brain in close proximity to a serotonergic fiber [23] makes it difficult to precisely ascertain the function of 5-HT in the brain, although one might suggest that 5-HT plays a modulatory role in CNS function.

8.2.2 Synthesis of 5-HT

Serotonin is an indolamine with a hydroxyl group at the 5 position and a terminal amine group on the carbon chain (Fig. 8.1). It is synthesized in serotonergic neurons from the essential amino acid tryptophan by two separate enzymes. The first, tryptophan hydroxylase (TPH), hydroxylates tryptophan using molecular oxygen, ascorbic acid, and biotin to synthesize 5-hydroxytryptophan (5-HTP), which is then decarboxylated into 5-HT by L-aromatic amino acid decarboxylase (AADC). TPH is the rate-limiting step in the production of 5-HT and is subject to both short-term and long-term regulatory processes. TPH can be directly activated by protein kinase A and by a calcium/calmodulin-dependent protein kinase, and the TPH gene promoter can be activated by cyclic adenosine monophosphate (cAMP) in vitro, all leading to a greater amount of 5-HT synthesis needed for heightened neurotransmission [24–26]. TPH activity can be regulated pharmacologically; 6-fluorotryptophan and *p*-fluorophenylalanine are short-lasting TPH inhibitors in vivo [27], whereas *p*-chlorophenylalanine is an irreversible inhibitor of TPH both in vivo and in vitro [28]. Inhibition of TPH results in a depletion of 5-HT and subsequent down-regulation of TPH and the 5-HT-transporter [29, 30].

In addition to TPH inhibition, 5-HT depletion can also be achieved with the use of halogenated amphetamines such as fenfluramine, formerly a prescribed appetite suppressant, and by the exclusion of tryptophan from the diet. The halogenated

amphetamines induce a rapid release of 5-HT from neurons by an unclear mechanism that may involve the disruption of the vesicular storage of the neurotransmitter resulting in an increase in the intracellular concentration of 5-HT and a reverse transport out of the nerve terminal [31, 32]. Amphetamine derivatives such as fenfluramine and methylenedioxymethamphetamine (MDMA) can also destroy 5-HT terminals leading to a long-lasting depletion of 5-HT [33, 34].

The exclusion of tryptophan from the diet can also reduce levels of 5-HT in the brain. For these studies, following a low-tryptophan diet, individuals are challenged with an amino acid-containing beverage that lacks tryptophan. These individuals display both a dramatic reduction of blood tryptophan levels and a greater than 90% reduction in 5-HT in the brain based on indirect measurements obtained by brain-imaging techniques [35]. This tryptophan depletion method has been used to study the role of 5-HT in psychiatric conditions and in the actions of psychotherapeutic agents [35]. Conversely, there are some reports that oral administration of L-tryptophan increases CNS 5-HT levels, most likely because TPH is not fully saturated [36–38]. Thus, the infusion of tryptophan has also been used experimentally to ascertain the roles of the 5-HT system in humans, though results have generally been less clear than with tryptophan depletion studies [35, 39].

8.2.3 Degradation and Reuptake of 5-HT

After release into the synaptic cleft, 5-HT is degraded, along with the catecholamine neurotransmitters, by monoamine oxidase type A (MAO_A). After 5-HT is oxidized by MAO_A, aldehyde dehydrogenase yields 5-hydroxyindoleacetic acid (5-HIAA) which is excreted in the urine. As such, many studies have measured levels of 5-HIAA in the cerebrospinal fluid (CSF), blood, and urine as a possible marker of central serotonergic function in psychiatric disorders. Most notably, decreased levels of 5-HIAA in the CSF is found in depressed patients who have recently attempted suicide [40, 41]. While the clinical utility of CSF measurements of 5-HT metabolites is limited, it has been shown that low levels of CSF 5-HIAA correlates with an increased risk of suicide following a known suicide attempt [42, 43]. In addition to MAO_A, serotonergic neurons express MAO_B intracellularly, though the affinity of this isoform for 5-HT is about 10-fold less [44]. Whether the B isoform significantly metabolizes intracellular 5-HT is unclear and it may be more likely that MAO_B is important for oxidation of other intracellular trace amines. MAO inhibitors such as phenelzine and tranylcypromine represent some of the earliest compounds useful in the treatment of depression, but despite their good efficacy, they are used less frequently today due to the risk of hypertensive crises and complex dietary restrictions.

In addition to the metabolism of extracellular 5-HT in the synaptic cleft, 5-HT is also actively transported back into the presynaptic terminal and recycled into vesicles. This reuptake of synaptic 5-HT is mediated by the 5-HT transporter (SERT), a 630-amino-acid protein with 12 transmembrane-spanning domains. SERT expression is almost entirely limited to serotonergic neurons as SERT mRNA is found in the raphe nuclei but is absent from other brain stem nuclei, while SERT protein expression is nearly ubiquitous in the CNS, consistent with the extensive nature of serotonergic projections [45–48]. SERT is extremely important clinically as the majority of antidepressants inhibit the activity of this transporter, thus prolonging serotonergic signaling. These include the selective 5-HT reuptake

inhibitors (SSRIs), such as fluoxetine, sertraline, and paroxetine, which are among the most widely prescribed antidepressants, as well as the older class of antidepressants known as tricyclics, such as amitriptyline and clomipramine. The SSRIs were given their name based on their ability to bind relatively selectively to SERT over the norepinephrine and dopamine transporters, in contrast to the tricyclic antidepressants which vary in their relative selectivities and thus inhibit the reuptake of multiple neurotransmitters, most notable norepinephrine. In addition to psychotherapeutic agents, several drugs of abuse interfere with SERT activity, along with other transporters. These include cocaine, amphetamines, and MDMA; the effects of these agents on SERT have been recently reviewed [49].

8.3 5-HT RECEPTORS

8.3.1 5-HT Receptor Nomenclature

The extraordinary diversity of CNS functions regulated by 5-HT underscores the complexity of the 5-HT system. This complexity is due, in part, to the vastness of the serotonergic projections from the raphe nuclei but is also due to the molecular diversity and differential cellular distribution of the multiple 5-HT receptor subtypes that are expressed in the brain. At least 14 different 5-HT receptors encoded by distinct genes have been identified since the advent of molecular cloning techniques and are divided into seven major classes: 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇ [50] (Fig. 8.2). Most of these classes have multiple subtypes, including the 5-HT₂ class that is divided into 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} [50, 51]. With the exception of 5-HT₃ receptors, which are ligand-gated ion channels [52, 53], 5-HT receptors are members of the G-protein-coupled receptor superfamily (Fig. 8.3).

Prior to molecular cloning, receptors were distinguished pharmacologically and as early as 1954 there was evidence of more than a single 5-HT receptor [54]. In 1957, Gaddum and Picarelli reported the existence of two distinct 5-HT receptor subtypes in the periphery which they called 5-HT_M and 5-HT_D based on their antagonists, morphine and dibenzyline, respectively [55]. In 1978, Leysen and colleagues discovered a serotonergic component of neuroleptic receptors labeled with [³H]spiperone [56], and further studies in rat brain homogenates revealed multiple 5-HT receptor subtypes in the CNS [16]. The 5-HT₁ receptor was labeled by [³H]5-HT, whereas the 5-HT₂ receptor (that corresponded to 5-HT_D) was labeled by the dopamine receptor ligand [³H]spiperone [16]. The M receptors were renamed 5-HT₃ receptors [57] and were found to be the only ionotropic subtype of 5-HT receptor [52, 58]. Soon after, heterogeneity of the 5-HT₁ receptor class became apparent as 5-HT_{1A} receptors could be distinguished pharmacologically from 5-HT_{1B} receptors (equivalent to the human 5-HT_{1D} receptor) [59]. A third type of 5-HT₁ receptor, the 5-HT_{1C} receptor, was demonstrated by autoradiographic techniques in the porcine choroid plexus through its high affinity for [³H]mesulergine and [³H]5-HT [60]. However, after the 5-HT_{1C} receptor was cloned, it was shown to possess 75% sequence homology to the 5-HT₂ receptor (now called 5-HT_{2A}) and, like the 5-HT₂ receptors, was also coupled to a phosphoinositol second messenger system instead of to the inhibition of adenylyl cyclase as are the members of the 5-HT₁ family [61]. The current 5-HT receptor nomenclature was adopted in 1994 and dictates that receptor classification should meet operational (drug-related characteristics), transductional (effector systems), and

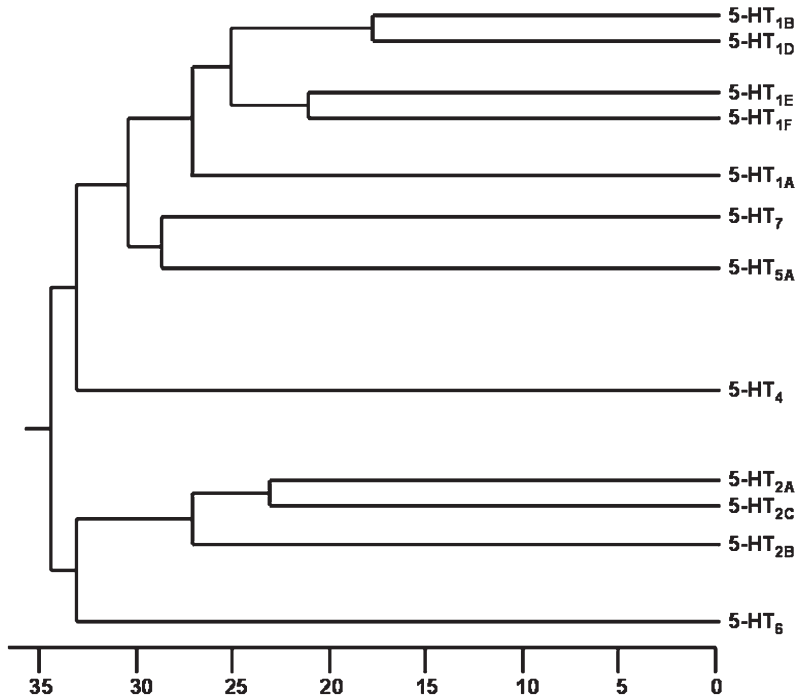


Figure 8.2 Dendrogram of 5-HT receptor subtypes. The relationships among the sequences of the various human 5-HT receptors are represented as a dendrogram. Groupings were calculated using the CLUSTAL algorithm [330] with the PAM 250 residue weight matrix. Horizontal axis indicates percentage difference among 5-HT receptors.

structural (genetic and amino acid sequence) criteria [62] (Fig. 8.2). As such, 5-HT receptors are now allocated to seven distinct families, 5-HT₁ to 5-HT₇, with multiple subtypes in some families (Table 8.1).

8.3.2 5-HT₁ Receptor Family

Among the seven classes of 5-HT receptors, the 5-HT₁ class is the largest and is comprised of five receptor types, with the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} characterized by a high affinity for 5-carboxamido-tryptamine (5-CT), the 5-HT_{1E} and 5-HT_{1F} characterized by a low affinity for 5-CT, and all five having a nanomolar affinity for 5-HT (Table 8.1). The original 5-HT_{1C} receptor is now classified as the 5-HT_{2C} receptor [61]. The genes encoding 5-HT₁ receptors have been cloned in both humans and rodents, allowing the demonstration that they all belong to the G-protein-coupled receptor superfamily with the characteristic seven hydrophobic transmembrane domains. All of the 5-HT₁ receptors interact with $G\alpha_i/G\alpha_o$ G proteins to inhibit adenylyl cyclase and modulate ion channels [63, 64]. Following is an overview of each member of the 5-HT₁ receptor family.

8.3.2.1 5-HT_{1A} Receptors. The 5-HT_{1A} receptors are particularly relevant to the antidepressant and anxiolytic responses in human beings. They are located presynaptically on serotonergic neurons in the raphe nuclei, where they act as

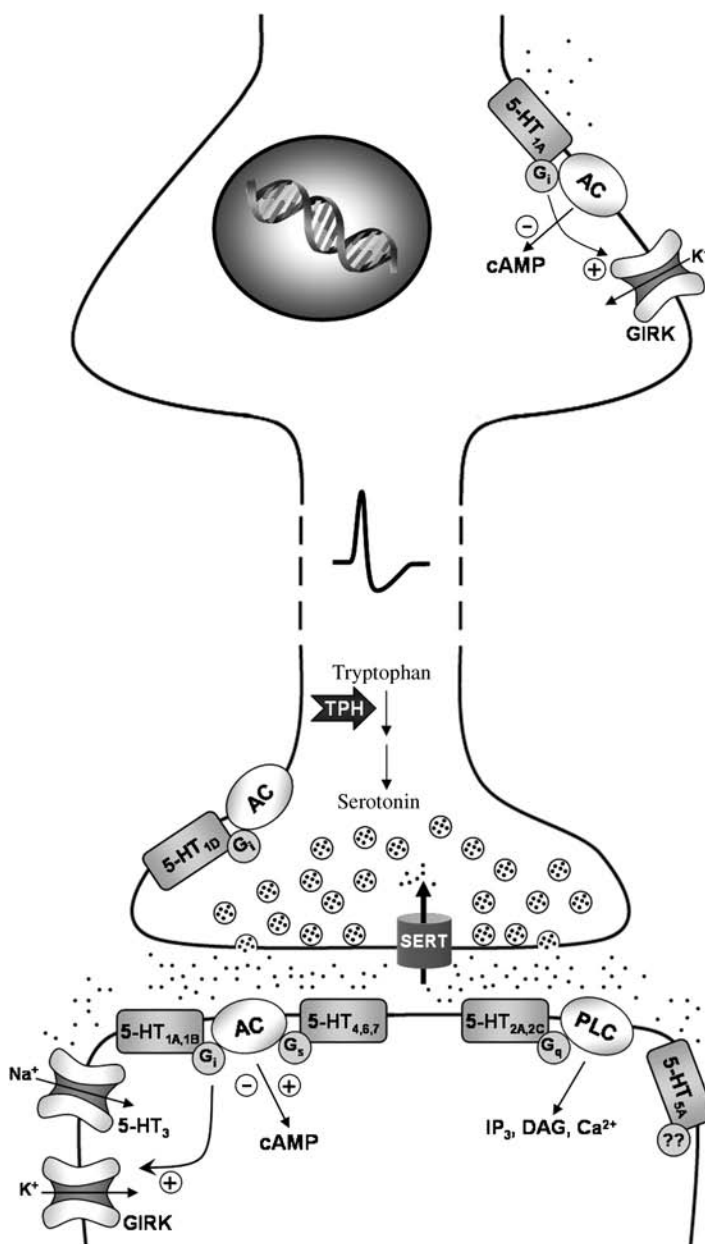


Figure 8.3 Model of a serotonergic neuron and synapse. Diagram of a serotonergic neuron originating from the brain stem raphe nuclei. 5-HT_{1A} receptors function both as autoreceptors on the serotonergic cell body and as postsynaptic receptors. Tryptophan and TPH are transported to the synapse where the synthesis of 5-HT is initiated. 5-HT_{1D} receptors function as autoreceptors on the presynaptic side of the synapse. Most 5-HT receptors are G-protein-coupled receptors and are located postsynaptically to serotonergic neurons. The 5-HT₃ receptor is the exception as it is a ligand-gated ion channel. The 5-HT_{1E} and 5-HT_{1F} receptors are also located postsynaptically and are coupled to G_i (not shown). The G-protein coupling of the 5-HT_{5A} receptor is still unclear. AC, adenylyl cyclase; PLC, phospholipase C; SERT, serotonin transporter; GIRK, G-protein-coupled inwardly rectifying potassium channels; TPH, tryptophan hydroxylase; IP₃, inositol trisphosphate; DAG, diacylglycerol.

TABLE 8.1 5-HT Receptors, Prototypical Ligands, and Clinical Use

Receptor	Prototypical Agonist	Prototypical Antagonist	Therapeutic Indications	Commonly Prescribed Medications and Drugs of Abuse
5-HT _{1A}	8-OH-DPAT	WAY100635	Anxiety, depression, neuroprotection	Buspirone (agonist); clozapine (partial agonist)
5-HT _{1B}	Sumatriptan	SB 224289	Anxiety, depression, migraine headache	Sumatriptan
5-HT _{1D}	Sumatriptan	BRL 15572	Migraine headache, depression	Sumatriptan
5-HT _{1E}	5-HT	None	Unknown	None
5-HT _{1F}	LY334370	None	Migraine headache	Sumatriptan
5-HT _{2A}	DOB	M100907	Schizophrenia, depression, anxiety, sleep disorders	LSD (agonist); atypical antipsychotic drugs and many antidepressants (antagonists)
5-HT _{2B}	BW 723C86	SB 204741	None	Pergolide, fenfluramine, and MDMA (agonists); clozapine (antagonist)
5-HT _{2C}	WAY-629	SB 206553	Appetite suppression	LSD (agonist); tricyclic antidepressants (antagonists)
5-HT ₃	None	Odansetron	Emesis	Odansetron
5-HT ₄	BIMU 8	SB 204070	Cognition enhancement, gastrointestinal motility	Cisapride
5-HT ₅	None	None	Unknown	LSD (agonist)
5-HT ₆	2-Ethyl-5-methoxy- <i>N,N</i> -dimethyltryptamine	MRS-245	Cognition enhancement	Many antidepressants and atypical antipsychotic drugs (antagonists)
5-HT ₇	None	SB 258719	Insomnia, depression	Antidepressants and many atypical antipsychotic drugs

autoreceptors to inhibit the firing rate of 5-HT neurons. The 5-HT_{1A} receptors are also located postsynaptically in limbic and cortical regions, where they may also attenuate firing [65] (Fig. 8.3). The highest densities of 5-HT_{1A} receptors in the brain are in the hippocampus, septum, amygdala, and cortical limbic areas whereas the 5-HT_{1A} receptors located in the raphe nuclei correspond to somatodendritic autoreceptors [66].

On serotonergic neurons, somatodendritic 5-HT_{1A} autoreceptors exert a negative-feedback influence on firing activity, when activated by an excess amount of 5-HT or by an exogenous agonist, by hyperpolarizing serotonergic neurons, thereby slowing down their baseline pacemaker firing activity [67–72]. Because 5-HT release is proportional to the firing rate of 5-HT neurons, excessive activation of 5-HT_{1A} autoreceptors results in a decrease of 5-HT release in projecting structures [73]. Likewise, postsynaptic 5-HT_{1A} receptors, which are particularly abundant in limbic structures, also have an inhibitory action on neuronal firing [72] (Fig. 8.3). Thus, the systemic administration of a 5-HT_{1A} receptor agonist, such as buspirone, results in a net decrease of 5-HT transmission at all postsynaptic 5-HT receptors, except those of the 5-HT_{1A} subtype.

The prototypic 5-HT_{1A} receptor agonist ligand is the aminotetralin derivative 8-OH-DPAT, though the azapirones probably represent the largest class of 5-HT_{1A} receptor ligands [74]. The azapirones are full agonists at 5-HT_{1A} autoreceptors and are generally, but not exclusively, partial agonists at postsynaptic 5-HT_{1A} receptors (Table 8.1). Effects of 5-HT_{1A} receptor-selective ligands on animal behavior have been extensively studied. Partial agonists (buspirone, ipsapirone, gepirone) and to a certain degree full 5-HT_{1A} receptor agonists (8-OH-DPAT) result in an anxiolytic-like effect [75, 76]. Of these drugs, only buspirone is clinically approved as an anxiolytic. With prolonged 5-HT_{1A} receptor agonist treatment, however, serotonergic neurons gradually recover their normal firing rate as a result of 5-HT_{1A} autoreceptor desensitization [67, 77], a process common among G-protein-coupled receptors [78]. This desensitization, however, is thought to be involved in the therapeutic action of 5-HT_{1A} receptor partial agonists as, like the SSRIs, there is a delay in the onset of therapeutic effects. The onset of action may actually be due to an increased activation of postsynaptic 5-HT_{1A} receptors occurring only after the 5-HT_{1A} autoreceptors are desensitized and the serotonergic neurons regain their normal level of firing activity. At this point, the summed effects of a normalized level of synaptic 5-HT and the exogenous 5-HT_{1A} agonist can be exerted on postsynaptic 5-HT_{1A} receptors.

In addition to anxiety, 5-HT_{1A} receptors may also be important in depression, aggression, obsessive-compulsive disorders, posttraumatic stress disorder, sexual behavior, appetite control, thermoregulation, and cardiovascular function [79–83]. Indeed, a deficiency in the 5-HT_{1A} receptor has been reproducibly found in the limbic system in mood disorders [83, 84], and further studies have indicated that low 5-HT_{1A} receptor levels may represent a risk factor in many psychiatric disorders [85]. As such, the generation of 5-HT_{1A} receptor knockout mice was of high priority and, in 1998, three groups simultaneously reported the generation of knockout mice in different genetic backgrounds [86–88]. All of the initial studies on the behavior of these 5-HT_{1A} receptor knockout mice found an anxiety-like phenotype in these mice. Importantly, anxiety was apparent not only in homozygote but also in heterozygote 5-HT_{1A} receptor knockout mice, indicating that a partial receptor deficit is sufficient to elicit the phenotype [86–88]. This suggested that transcriptional and posttranslation

mechanisms of 5-HT_{1A} receptor regulation, such as receptor downregulation, may contribute to the pathogenesis of some psychiatric disorders. For additional review of 5-HT_{1A} receptor knockouts as a model for anxiety, see Toth, 2003 [85].

8.3.2.2 5-HT_{1B} and 5-HT_{1D} Receptors. Due to the considerable similarity between 5-HT_{1B} and 5-HT_{1D} receptors, they will be discussed together. Indeed, the similarity between these receptors and interspecies differences has resulted in a complicated history. As such, the 5-HT_{1B} receptor was initially claimed to exist only in rodents (rat, mouse, and hamster) [59, 65], whereas in humans, two distinct receptors had been cloned and were named 5-HT_{1D α} and 5-HT_{1D β} [89, 90]. The human 5-HT_{1D α} and 5-HT_{1D β} receptors display about 77% sequence homology and their pharmacological properties are nearly indistinguishable [90]. The subsequent cloning of the rodent 5-HT_{1B} receptor, however, revealed that the 5-HT_{1B} receptor was homologous to the human 5-HT_{1D β} receptor [90–94]. The same nomenclature is now recommended for this receptor in all mammalian species; thus, the 5-HT_{1D β} receptor in humans was renamed h5-HT_{1B}, and the rat 5-HT_{1B} receptor, r5-HT_{1B} [93, 94]. Likewise, the human 5-HT_{1D α} receptor was renamed 5-HT_{1D} [93, 94]. There are, interestingly, a few key pharmacological differences between the rodent and human 5-HT_{1B} receptors. For example, some β -adrenergic antagonists, such as (–)propranolol, bind to r5-HT_{1B} receptors with a much higher affinity than to h5-HT_{1B} receptors [90, 91] due to single-amino-acid difference (asparagine vs. threonine) in the seventh transmembrane domain of the receptors [95, 96].

The 5-HT_{1B} receptor binding sites have been detected by autoradiography and immunohistochemistry with high densities in the basal ganglia, particularly in the globus pallidus and substantia nigra [66, 97–99]. The distribution of the 5-HT_{1B} receptor binding sites found in rat brain was similar to that of the 5-HT_{1D β} receptor binding sites observed in human [98, 100–102]. The 5-HT_{1B} receptor binding sites are also found at lower densities in the cerebral cortex, amygdala, hypothalamus, and spinal cord [66, 99]. The 5-HT_{1B} receptors are predominantly found at the presynaptic terminal where they can function as both autoreceptors on serotonergic neurons and heteroreceptors on nonserotonergic neurons to modulate neurotransmitter release [103–109] (Fig. 8.3). Thus, activation of 5-HT_{1B} receptors has been reported to inhibit the release of 5-HT in the hippocampus and frontal cortex [110–112]. Moreover, as heteroreceptors, 5-HT_{1B} receptor activation has been shown to have an inhibitory effect on the release of γ -aminobutyric acid (GABA), acetylcholine, glutamate, and dopamine [113–115]. Indeed, while 5-HT_{1B} receptors have been traditionally associated with 5-HT autoreceptor function, it is becoming clear that most of the 5-HT_{1B} receptors in the brain are expressed on nonserotonergic neurons [113].

The 5-HT_{1B} receptors have been shown to be involved in several physiological functions, behaviors, and psychiatric diseases, including locomotor activity, drug abuse reinforcement, migraine, anxiety states, and aggressive behavior [116–121] (Table 8.1). For example, 5-HT_{1B} receptor knockout mice were reported to exhibit increased aggressive behavior toward other mice in resident–intruder aggression tests [121, 122] and have also been reported to show decreased anxiety and behavioral hyperactivity manifested as increased exploratory behavior [122, 123]. This increased aggressive behavior and hyperactivity may suggest that 5-HT_{1B} receptors play a role in impulse control. The findings with the 5-HT_{1B} receptor knockout mice correlate

with the findings that certain 5-HT_{1B} receptor agonists (serenics) have antiaggressive properties [124]. The 5-HT_{1B} receptor has also been implicated in the reinforcing and aversive actions of cocaine in rodents by both pharmacological and null mutation strategies [125]. For example, 5-HT_{1B} receptor knockout mice have also shown increased vulnerability and altered responses to several drugs of abuse such as increased voluntary self-administration of cocaine and increased locomotor response to cocaine [126]. In addition, several investigators have reported increased ethanol consumption in 5-HT_{1B} receptor knockout mice [127, 128]. Interestingly, behavior studies have also demonstrated that 5-HT_{1B} receptor agonists induce a learning deficit [129] whereas a 5-HT_{1B} receptor antagonist enhances learning consolidation [130]. The precise role of the 5-HT_{1B} receptor, however, remains largely unknown and is complicated by the lack of selective pharmacological agents and by the contradictory effects of 5-HT_{1B} autoreceptors and heteroreceptors.

Like the 5-HT_{1B} receptor, the ultimate clinical significance of the 5-HT_{1D} receptor remains largely unknown again due to the lack of selective drugs (Table 8.1). There has been speculation, however, that these receptors might be involved in anxiety, depression, and other neuropsychiatric disorders, but this remains, for the most part, unsubstantiated [113]. Another complication is that studies in all species are faced by the low levels of the 5-HT_{1D} versus 5-HT_{1B} receptor in brain [99]. Studies using nonselective radioligands in the presence of more selective inhibitors of other receptor sites have suggested that 5-HT_{1D} receptors are located in various brain regions but at higher levels in the basal ganglia but also in the hippocampus and cortex [99, 131]. There is also clear evidence from receptor autoradiography and in situ hybridization studies that the 5-HT_{1D} receptor, like the 5-HT_{1B} receptor, is located presynaptically on both serotonergic and nonserotonergic neurons [131]. Thus, 5-HT_{1D} receptors may also have autoreceptor and heteroreceptor actions on the inhibition of neurotransmitter release (Fig. 8.3).

The 5-HT_{1D} receptors have been implicated in the pathophysiology of migraine because 5-HT_{1D} receptor agonists, such as sumatriptan, have remarkable efficacy in treating migraine. However, there is considerable controversy regarding the nature of the actual 5-HT receptors involved in migraine since sumatriptan binds nearly equally well at 5-HT_{1D} and 5-HT_{1B} receptors [132] and also binds to 5-HT_{1F} sites [133]. It has been suggested that 5-HT_{1B} receptors may be primarily involved in neurogenic inflammation [134] due to their preponderance in neuronal tissue, whereas 5-HT_{1D} receptors may be more involved in vasoconstriction [135].

8.3.2.3 5-HT_{1E} Receptors. The 5-HT_{1E} receptor was first detected in radioligand binding studies using [³H]5-HT while masking the other 5-HT₁ subtypes known at that time (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}), leading to a biphasic displacement curve to 5-CT [136, 137]. One component of these curves was associated with 5-HT_{1D} binding, whereas the other was attributed to a novel receptor, the 5-HT_{1E} receptor [137]. Later, the 5-HT_{1E} gene was cloned by several independent laboratories from human genomic libraries [138, 139] and a human complementary deoxyribonucleic acid (cDNA) library [140] and found to have a pharmacology similar to that described by Teitler's group [137].

The protein distribution of 5-HT_{1E} receptor in the brain is inconclusive because no selective ligands or antibodies are available, though autoradiographic studies have been performed while masking other 5-HT₁ receptors [99, 141, 142]. These studies

indicate that in all species higher levels of these binding sites were present in the cortex, caudate, and putamen, but detectable levels were found in other areas, including hippocampus and amygdala. The 5-HT_{1E} receptors appear to have a postsynaptic location [142].

Currently, nothing is known about the physiological function of the 5-HT_{1E} receptor. This is partly due to the unavailability of selective pharmacological tools, but the lack of an animal model also makes it hard to explore the function of this receptor (Table 8.1). A high level of expression of 5-HT_{1E} receptor messenger ribonucleic acid (mRNA) in the hippocampus and olfactory [143] may suggest a role for this receptor in the regulation of memory, learning, and emotion [144, 145].

8.3.2.4 5-HT_{1F} Receptors. The newest 5-HT₁ receptor to be cloned is the human 5-HT_{1F} receptor. This 5-HT_{1F} receptor gene was originally detected in the mouse on the basis of its sequence homology with the 5-HT_{1B} and 5-HT_{1D} receptor subtypes [146]. The human 5-HT_{1F} gene followed shortly afterward [133]. Initial studies located 5-HT_{1F} mRNA in the mouse and guinea pig brain using in situ hybridization with an abundance in the hippocampus, cortex, and dorsal raphe nucleus [133, 146, 147]. Autoradiography studies using [³H]sumatriptan in the presence of 5-CT demonstrated a good correlation with the distribution of 5-HT_{1F} mRNA. [131, 148–150]. The brain distribution of 5-HT_{1F} binding sites labeled by a novel, selective 5-HT_{1F} radioligand, [³H]LY334370, was reported and demonstrated a low abundance and fit with the restricted distribution of this receptor subtype [151].

The clinical significance of 5-HT_{1F} receptors is unknown at this time. The binding of sumatriptan at this receptor population suggests that 5-HT_{1F} receptors may be involved in migraine [133], and a single clinical trial showed that a 5-HT_{1F} agonist had antimigraine actions, although the compound was later dropped due to toxicity (D. Nelson, personal communication) [152] (Table 8.1). Recent studies show that 5-HT_{1D} receptors are the dominant species in human cerebral blood vessels, but they further show that 5-HT_{1F} receptors are expressed in both neural and vascular tissue [134, 135].

8.3.3 5-HT₂ Receptor Family

The 5-HT₂ receptor family consists of three receptor subtypes, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, which are similar in terms of their molecular structure, pharmacology, and signal transduction pathways [51]. The 5-HT_{2A} receptor was originally designated as the 5-HT_D receptor and later the 5-HT₂ receptor [16, 55, 62]. The original 5-HT_{1C} receptor was renamed the 5-HT_{2C} receptor after it was cloned because of significant homology and coupled effector systems as the 5-HT_{2A} receptor [61]. Another 5-HT₂-like receptor, based on similar pharmacology, was found in the stomach fundus [153, 154] and when cloned it was found to be structurally related to the 5-HT_{2A} and 5-HT_{2C} receptors and was thus named 5-HT_{2B} [155, 156]. The members of the 5-HT₂ family of receptors are all G-protein-coupled receptors and have high degree of homology within the seven transmembrane domains but are structurally distinct from other 5-HT receptors [157]. The 5-HT₂ receptors interact primarily with the G α_q class of G proteins to result in the activation of phospholipase C and the mobilization of intracellular calcium [158], and stimulation of these receptors causes cell excitation.

8.3.3.1 5-HT_{2A} Receptors. The distribution of 5-HT_{2A} receptors in the CNS has been extensively characterized by autoradiography, in situ hybridization, and immunohistochemistry. Receptor autoradiography studies using [³H]-spiperone, [³H]-ketanserin, [¹²⁵I]-DOI, and [³H]-MDL 100907 as radioligands have demonstrated high levels of 5-HT_{2A} receptor binding sites in many forebrain regions, particularly various cortical regions, with lower levels in the basal ganglia and hippocampus [159–162]. There has generally been close concordance between the distribution of 5-HT_{2A} receptor binding sites, mRNA, and receptorlike immunoreactivity [163–168], suggesting that the cells expressing 5-HT_{2A} receptors are located in the region where the receptors are present and are thus located postsynaptically relative to the serotonergic neuron (Fig. 8.3).

The 5-HT_{2A} receptors are particularly abundant in the pyramidal neurons primarily from cortical layers IV and V [164, 165, 168]. Within the cortical pyramidal neurons, 5-HT_{2A} receptors are preferentially sorted to the apical dendrites [164, 165, 168] where information from the dendritic tree integrates before descending onto the soma and into the axon [169]. The 5-HT_{2A} receptors in the apical dendrites mediate the 5-HT-induced enhancement of excitatory postsynaptic potential (EPSP) in pyramidal neurons, which may be responsible for the psychotomimetic effects of some hallucinogens [170, 171].

The 5-HT_{2A} receptors are essential for mediating a large number of physiological processes in the periphery and in the CNS, including platelet aggregation, smooth muscle contraction, and modulation of mood and perception [51]. Many drugs of diverse therapeutic classes mediate their actions, at least in part, by interactions with 5-HT_{2A} receptors. Most but not all hallucinogens [172], including LSD and *N,N*-dimethyltryptamine, function as agonists at 5-HT_{2A} receptors [173], while all clinically approved atypical antipsychotic drugs are potent 5-HT_{2A} receptor antagonists [174] (Table 8.1). In vivo studies have demonstrated that 5-HT_{2A} receptors mediate 5-hydroxytryptophan-induced head twitch behavior [175–179], wet dog shakes [176, 180], and urinary bladder contraction [181]. It was shown that the potency with which 5-HT₂ receptor antagonists inhibit agonist-induced head shakes closely correlated with their affinity for the 5-HT_{2A} receptor binding site but not other binding sites, including the 5-HT_{2C} receptor binding site [178, 182]. Furthermore, 5-HT_{2A} receptor-selective antagonists such as MDL 100907 inhibited the head shake response while 5-HT_{2B} and 5-HT_{2C} receptor-selective antagonists (SB 200646A) did not [178, 183]. In humans, a direct linear correlation has been demonstrated between the abilities of a variety of hallucinogens to induce hallucinations and their abilities to bind to 5-HT_{2A} receptors [184]. Indeed, the actions of psilocybin in humans can be blocked by either ketanserin or risperidone but not by haloperidol [185], indicating that the actions of hallucinogens in humans are mediated via activation of 5-HT_{2A} receptors.

The importance of 5-HT_{2A} receptors in schizophrenia was recognized as early as 1954 [10, 54] with further support coming with the discovery that reserpine, a drug with some efficacy in treating schizophrenia, depletes 5-HT [11, 12] and the demonstration of a serotonergic component of antipsychotic drug binding in 1978 [56]. The discovery that clozapine, a drug highly effective in treating schizophrenia [186], is a 5-HT_{2A} receptor antagonist [187] that downregulates 5-HT_{2A} receptors [188, 189] further reinforced the hypothesis that 5-HT_{2A} receptor blockade may be beneficial in schizophrenia. It was soon discovered that atypical antipsychotic drugs, as a group, bound with higher affinity to 5-HT_{2A} receptors than to dopamine

D₂ receptors [190]. Indeed, atypical antipsychotic drugs can be classified based on a 5-HT_{2A}-D₂ affinity ratio of >1, while the typical antipsychotic drugs all have 5-HT_{2A}-D₂ affinity ratios <1 [174]. Interestingly, risperidone and olanzapine, both of which have high 5-HT_{2A} receptor blocking activity relative to their D₂ affinity, have greater efficacy for treating negative symptoms of schizophrenia and produce fewer extrapyramidal side effects than haloperidol [191, 192]. Selective 5-HT_{2A} receptor antagonists (especially MDL 100907 and SR46349B) have shown promise in animal models to be predictive of atypical antipsychotic action [193] and have demonstrated efficacy in treating schizophrenia [194]. Additional evidence highlighting the role of 5-HT_{2A} receptors in schizophrenia comes with evidence of an association between schizophrenia and treatment outcome and certain polymorphic variants of the 5-HT_{2A} receptor [195]. To our knowledge selective 5-HT_{2A} antagonists represent the only known clinically effective antipsychotic drugs which do not have appreciable affinity for dopamine D₂ receptors [2].

More recently, 5-HT_{2A} receptors have been implicated as mediating viral entry of the JC virus—the causative agent of progressive multifocal leukoencephalopathy (PML) [196]. In these studies, the JC virus was shown to require intact 5-HT_{2A} receptors for viral entry into neuronal cells and 5-HT_{2A}-selective antagonists were able to block viral entry. These results imply that 5-HT_{2A} antagonists may represent novel treatments for PML, which is the main cause of human immunodeficiency virus (HIV) dementia [197].

8.3.3.2 5-HT_{2B} Receptors. The 5-HT_{2B} receptors are found in many organs, including the stomach fundus [198], vascular smooth muscle [199], heart valves [200], spinal cord [201], and at low levels in some brain regions [202]. Indeed, the presence of the 5-HT_{2B} receptors in the brain has been controversial, but it is now clear that 5-HT_{2B} receptor mRNA and protein are expressed with very limited distribution but are potentially of functional importance [153, 203–205]. By immunohistochemistry, 5-HT_{2B} receptor–like immunostaining was reported to be limited to only a few brain regions, particularly the cerebellum, lateral septum, dorsal hypothalamus, and medial amygdala [204]. In this study, the cells expressing 5-HT_{2B} receptor–like immunoreactivity have a neuronal and not astrocytic morphology [204].

There are little available data on the functional effects of activation of the central 5-HT_{2B} receptor. It has been suggested, however, that the 5-HT_{2B} receptor has a role in anxiety as the nonselective 5-HT₂ receptor agonist BW723C86 is reported to have anxiolytic properties in the rat social interaction test that can be reversed by a selective 5-HT_{2B} receptor antagonist [206, 207]. Interestingly, BW723C86 has an anxiolytic effect when injected directly into the medial amygdala [208], which contains detectable amounts of 5-HT_{2B} receptor–like immunoreactivity [204]. Outside of the CNS, activation of the 5-HT_{2B} receptor has recently been shown to mediate the valvulopathic side effects of the now-banned appetite suppressant fenfluramine [209] (Table 8.1). It is likely that other pharmaceuticals and drugs of abuse may activate 5-HT_{2B} with potential to lead to valvular heart disease [210].

8.3.3.3 5-HT_{2C} Receptors. The 5-HT_{2C} receptor was originally identified as a [³H]5-HT binding site in the choroid plexus of various species that could also be labeled by [³H]mesulergine and [³H]LSD but not by [³H]ketanserin [60]. Originally this site was named the 5-HT_{1C} receptor because of its high affinity for [³H]5-HT

[211] but was reclassified as the 5-HT_{2C} receptor after the receptor was cloned and further characterized [212]. The mouse 5-HT_{2C} receptor was partially cloned in 1987 [213], and this was shortly followed by the sequencing of full-length clones in rat, mouse, and human [61, 214, 215]. Unlike the 5-HT_{2A} and 5-HT_{2B} receptors, there is very little 5-HT_{2C} receptor expression outside of the CNS. Autoradiographic studies have provided a detailed map of 5-HT_{2C} receptor binding sites in rat and other species [216–218]. In addition to the very high levels in the choroid plexus, 5-HT_{2C} receptor binding sites are widely distributed in the cortex, basal ganglia, hippocampus, and hypothalamus [217]. Due to correlation between 5-HT_{2C} receptor mRNA and receptor binding sites, the 5-HT_{2C} receptor is clearly located postsynaptically [166, 219] (Fig. 8.3).

The 5-HT_{2C} receptors have been shown to undergo a form of posttranslational regulation known as RNA editing [220]. RNA editing in mammalian systems generally involves the conversion of adenosine residues to inosines at the mRNA level [221] and has the potential to result in multiple protein isoforms that may have altered functions. Editing of the 5-HT_{2C} receptor was discovered by comparing sequences from genomic DNA and cDNAs from the rat striatum [220] which showed five different adenosines that could be edited and predicted the formation of protein isoforms with up to three amino acids changed in the second intracellular loop of the receptor [222–224]. The genomic DNA predicted isoleucine, asparagine, and isoleucine at positions 157, 159, and 161 (5-HT_{2C-INI}), whereas the cDNA library predicted valine, serine, and valine at those positions (5-HT_{2C-VSV}). Further analysis of cDNA sequences isolated from rat brain revealed the tissue-specific expression of 7 major 5-HT_{2C} receptor isoforms that are encoded by 11 distinct RNA species [224]. Because the second intracellular loop has been implicated in receptor activation and G-protein coupling [225, 226], it is likely that these isoforms have different intracellular signaling properties. Indeed, many studies have demonstrated altered signaling properties of 5-HT_{2C}-editing isoforms [227]. The *in vivo* functional consequences of RNA editing of the 5-HT_{2C} receptor are unknown and create a challenge of isolating the function of a single, specific isoform, although several studies have suggested alterations in RNA editing in various psychiatric illnesses, including schizophrenia [228] and suicide [229], and by stress and antidepressant administration [230].

Both pharmacological and genetic approaches to the analysis of 5-HT_{2C} receptor function have revealed that it is involved in the regulation of a wide range of behavioral and physiological processes, including regulation of the dopaminergic system and regulation of appetite, and plays a role in drug abuse, anxiety, and depression [231] (Table 8.1). Activation of 5-HT_{2C} receptors has been shown to have significant inhibitory effects on both the limbic and striatal dopamine pathways that may contribute to the efficacy or side-effect profile of dopaminergic agents such as antipsychotics [232–236]. Indeed, many antipsychotic drugs have high affinities for 5-HT_{2C} receptors [237] where they function as inverse agonists [238]. It is also possible that the influence 5-HT_{2C} receptor agents have on mesolimbic dopamine transmission could modulate the reinforcing and behavioral effects of drugs of abuse [239–241]. The 5-HT_{2C} receptors also contribute substantially to the serotonergic suppression of feeding as 5-HT_{2C} agonists produce hypophagia that is blocked by antagonists [242, 243]. In addition, 5-HT_{2C} receptor knockout mice were found to have elevations of body weight and adiposity [244] likely related to chronically increased food intake [245]. Indeed, selective 5-HT_{2C} receptor agonists have shown promise as anorectic

agents in humans [246] and many 5-HT_{2C} receptor-selective agonists which avoid interactions with 5-HT_{2B} receptors are currently in testing.

8.3.4 5-HT₃ Receptors

In contrast to the other known 5-HT receptors that are all members of the G-protein-coupled receptor superfamily, the 5-HT₃ receptor belongs to the superfamily of ligand-gated ion channels. Responses mediated by 5-HT₃ receptors have been studied since the 1950s, but due to the lack of pharmacological approaches to study this receptor, it was not until recently that the 5-HT₃ receptor was appreciated to be the originally described 5-HT_M receptor [52, 55, 247, 248]. The 5-HT₃ receptor channel complex, like other members of this superfamily, is a pentameric assembly of individual protein subunits, where each subunit is thought to have four transmembrane domains [249]. When the first gene encoding the 5-HT₃ receptor (the 5-HT_{3A} receptor subunit) was cloned and expressed in heterologous expression systems, it formed functional homomeric channels [52]. A shorter splice variant of the 5-HT_{3A} receptor subunit has also been cloned from mouse, rat, and guinea pig and was shown to be the most abundant, and in humans the only, form in the CNS [247, 248, 250]. However, no functional differences between these alternatively spliced 5-HT_{3A} receptor subunits have been found to date [251]. In 1998, a second 5-HT₃ receptor subunit, the 5-HT_{3B} receptor subunit, was cloned [53, 252]. This subunit does not form functional channels on its own but requires coassembly with the 5-HT_{3A} receptor subunit for its function, yielding a 5-HT₃ receptor with significantly larger single-channel conductance [53, 253]. There is currently conflicting lines of evidence concerning whether the 5-HT_{3B} subunit is expressed in the CNS forming heteromeric receptor complex [251].

The 5-HT_{3A} receptor subunit is ubiquitously expressed in the CNS at low levels, and 5-HT₃ receptors are thought to participate in a variety of physiological processes, including cognitive processing [249]. In the CNS, 5-HT₃ receptors are expressed in the cortex, hippocampus and amygdala, medulla oblongata, spinal cord, and to a lesser extent nucleus accumbens, striatum, and substantia nigra [254]. On a cellular level, postsynaptic 5-HT₃ receptors mediate fast excitatory synaptic transmission in rat neocortical interneurons and amygdala [255, 256] and are also present on presynaptic nerve terminals, where they are thought to mediate or modulate neurotransmitter release [257, 258] (Fig. 8.3). The 5-HT₃ receptor antagonists, such as ondansetron, are clinically effective in the treatment of chemotherapy-induced or radiation-induced nausea and vomiting [259], whereas they are ineffective against motion sickness and apomorphine-induced emesis [260] (Table 8.1). Preclinical studies have suggested that 5-HT₃ receptor antagonists may also enhance memory and be of benefit in the treatment of anxiety, depression, and dementia [260]. One of the most attractive features of 5-HT₃ antagonists is their general lack of undesirable side effects characteristic of many psychotherapeutic agents. Very little is known about the possible therapeutic application of 5-HT₃ agonists, however, though it seems that some partial agonists may be anxiolytic [261].

8.3.5 5-HT₄ Receptors

The 5-HT₄ receptor was initially identified in cultured neurons from mouse colliculi and guinea pig brain by stimulation of adenylyl cyclase activity [262, 263]. This was

thus the first 5-HT receptor shown to couple positively to adenylyl cyclase, though the ability of 5-HT to stimulate adenylyl cyclase had been known for a number of years prior [264, 265]. Several splice variants of the 5-HT₄ receptors have been cloned, differing in the length of their C-terminal tails [266, 267]. In heterologous expression systems, these receptors present some functional and pharmacological differences. In particular, the length of the C-terminal sequence of the receptor was shown to be implicated in its constitutive activity [268].

The 5-HT₄ receptors have a broad tissue distribution with important roles in both the CNS and the periphery [269–272]. In the brain, 5-HT₄ receptors are localized postsynaptically on neurons and may mediate slow excitatory responses to 5-HT (Fig. 8.3). In collicular and hippocampal neurons, 5-HT₄ receptors stimulate adenylyl cyclase and an inhibition of K⁺ channels mediated by the activation of cAMP-dependent protein kinase [273]. Peripherally, 5-HT₄ receptors facilitate acetylcholine release in guinea pig ileum and may play a role in the peristaltic reflex [269]. Consequently, 5-HT₄ receptors have been implicated in a variety of pathological disorders; in particular, 5-HT₄ receptor agonists show efficacy in the treatment of irritable bowel syndrome [274] (Table 8.1). In addition, 5-HT₄ receptor agonists have shown promise in the improvement of cognitive function by enhancing cholinergic transmission in the hippocampus [1, 275, 276]. As such, 5-HT₄ receptors may be involved in memory and learning and have been shown to be markedly decreased in patients with Alzheimer's disease [113, 277].

8.3.6 5-HT₅ Receptor Family

The 5-HT₅ receptor family is a poorly understood class of 5-HT receptors that consists of two members designated as 5-HT_{5A} and 5-HT_{5B}. Interestingly, only the 5-HT_{5A} receptor is expressed in humans as the 5-HT_{5B} receptor coding sequence is interrupted by stop codons [278, 279]. Both the 5-HT_{5A} and 5-HT_{5B} receptors are, however, expressed in mouse and rat [280–283]. The 5-HT₅ receptors exhibit less than 50% amino acid sequence homology with other 5-HT receptors, suggesting that they represented a distinct family of receptors and early attempts failed to demonstrate G-protein coupling [280–283] (Fig. 8.3). However, subsequent studies have shown that 5-HT_{5A} receptors can signal via G α_i /G α_o proteins and the inhibition of adenylyl cyclase in heterologous expression systems [284]. Interestingly, 5-HT_{5A} receptors also appear to be coupled to the inhibition of adenosine diphosphate (ADP)–ribosyl cyclase and the activation of phospholipase C, suggesting a complex crosstalk between multiple signal transduction pathways [285, 286]. In situ hybridization studies demonstrated a low-level but widespread distribution of 5-HT_{5A} receptor mRNA in neuronal cells in both mouse and rat brain [280, 282]. It appears, however, that the primary site of 5-HT_{5A} receptor expression is nonneuronal [284].

The pharmacological function of 5-HT_{5A} receptors is currently unknown though it has been speculated that, on the basis of their localization, they may be involved in motor control, feeding, anxiety, depression, learning, memory consolidation, adaptive behavior, and brain development [278, 281, 283] (Table 8.1). The 5-HT_{5A} receptors may also be involved in a neuronally driven mechanism for regulating astrocyte physiology, with relevance to gliosis, and thus may play a role in the pathophysiology of diseases such as Alzheimer's disease [284]. A 5-HT_{5A} receptor knockout mouse line has been made with minimal phenotypic changes observed to date [287]. These mice do, however, display increased locomotor activity and

exploratory behavior, though there was no difference in the anxietylike behavior of these animals in the elevated plus maze paradigm [287].

8.3.7 5-HT₆ Receptors

The 5-HT₆ receptor was initially detected by two groups following identification of a cDNA sequence which encoded a 5-HT-sensitive receptor with a novel pharmacology [288, 289] and was followed by the cloning human 5-HT₆ receptor [291]. The 5-HT₆ receptor can be radiolabeled with [¹²⁵I]LSD and couples to the stimulation of adenylyl cyclase [288, 289]. The 5-HT₆ receptor mRNA appears largely confined to the CNS, though low levels have been detected in the stomach and adrenal glands [288, 289]. Within the brain, high levels of 5-HT₆ receptor mRNA are found in the striatum, nucleus accumbens, and hippocampus [288–292]. By immunohistochemistry, 5-HT₆ receptor-like immunoreactivity was abundant in similar regions as the 5-HT₆ receptor mRNA (striatum, nucleus accumbens, hippocampus, and cerebral cortex), suggesting that the receptor protein is expressed in close proximity to the site of synthesis [291]. Further studies revealed that 5-HT₆ receptors are predominantly postsynaptic to serotonergic neurons and are not autoreceptors [291–294] (Fig. 8.3).

The distribution of the 5-HT₆ receptor as well as its high affinity for a number of antipsychotics, including clozapine and loxapine, and some tricyclic antidepressants such as amoxipine, amitriptyline, and clomipramine [288, 295, 296] has led to significant efforts to understand its possible role in psychiatry. The first behavioral studies of 5-HT₆ receptor function used antisense oligonucleotides to decrease the level of 5-HT₆ receptor expression [297]. In these studies, rats exhibited an increased number of yawns and stretches that could be blocked by atropine, suggesting a role of the 5-HT₆ receptor in the control of cholinergic neurotransmission [298, 299]. Thus, 5-HT₆ antagonists may have a role in the treatment of cognitive dysfunction such as in Alzheimer's disease [1, 300]. Indeed, the selective 5-HT₆ antagonist SB-271046 has been shown to improve memory retention in the water maze test of spatial learning and memory [299, 301] (Table 8.1). The 5-HT₆ receptors also appear to have a role in anxiety as a reduction in 5-HT₆ receptor levels by antisense oligonucleotides suppressed 5-HT release in a conditioned fear stress paradigm [302]. In addition, a 5-HT₆ knockout mouse demonstrated a reduced response to the acute effects of ethanol [303]. It is important to point out, however, that 5-HT₆ receptor knockout mice may be of limited utility as the levels of 5-HT₆ receptor expression in mice is very low and there are significant pharmacological differences between the mouse 5-HT₆ receptor and the rat and human receptors [304, 305]. It appears likely, though, that 5-HT₆ receptors will have an important future role in the treatment of neuropsychiatry illnesses—especially in terms of enhancing cognition.

8.3.8 5-HT₇ Receptors

In 1993, the 5-HT₇ receptor was cloned independently by researchers in four laboratories [306–309]. This G-protein-coupled receptor protein was found to be positively coupled to adenylyl cyclase through G α_s [306–309]. The 5-HT₇ receptor mRNA was found in the brain, mainly in the hypothalamus, thalamus, hippocampus, and cortex, and in the periphery, mainly in blood vessels and the intestines [306–309]. At least three different splice variants of the 5-HT₇ receptor have been identified in rat

and human tissues, though the functional role of these splice variants is still unknown [310–312]. It is possible that since these variants differ in the length of their C-terminal tails and in the number of phosphorylation sites, they are likely to have functional differences in their susceptibility to desensitize and the efficiency of G-protein coupling.

The 5-HT₇ receptor exhibits a distinct distribution in the CNS with both mRNA and receptor binding sites displaying a similar pattern of distribution with relatively high levels in the thalamus, hypothalamus, and hippocampus and lower levels in the cortex and amygdala [313–316]. Significant tissue-specific differences in the distribution of 5-HT₇ receptor splice variants is not apparent [310, 311, 317], though the overall abundance of individual isoforms differs among species [310, 311, 317]. A low level of expression of 5-HT₇ receptors has been detected in the periphery, especially in the smooth muscle of blood vessels [318, 319].

Recent studies suggest that the 5-HT₇ receptor is involved in thermoregulation, circadian rhythm, learning and memory, hippocampal signaling, sleep, and endocrine regulation. For example, in rat hippocampal slices, 8-OH-DPAT induced a phase resetting within the suprachiasmatic nucleus (a region important in the regulation of circadian rhythms) that was shown to be mediated by the 5-HT₇ receptor [308, 320–323]. In addition, selective 5-HT₇ receptor antagonists administered to rats at the beginning of the sleep phase increased the latency to rapid eye movement (REM) sleep and decreased the amount of time spent in REM sleep [324], changes that are directly opposite of the sleep patterns seen in depressed patients. Several antipsychotics and antidepressants have high affinities for the 5-HT₇ receptor [289, 296], and antidepressants may exert some of their function through the 5-HT₇ receptors [325]. These findings are compatible with the hypothesis that the 5-HT₇ receptor is of considerable importance for regulating sleep, circadian rhythms, and mood.

The 5-HT₇ receptor has also been shown to have an important role in hippocampus-dependent functions such as learning and memory [326]. For example, 5-HT₇ receptor knockout mice have been found to exhibit a specific impairment in contextual fear conditioning in which the animal learns to associate the environment with an aversive stimulus and is generally believed to depend on the hippocampus [327]. There was no difference, however, between wild-type and knockout mice in the Barnes maze test, in which the mouse must learn how to escape an open area by locating a chamber using environmental cues [327]. Electrophysiological studies have shown that 5-HT₇ receptor activation decreased the amplitude of slow afterhyperpolarizations in the CA3 region of the hippocampus by inhibiting Ca²⁺-activated K⁺ channels [50a] and to modulate the excitability and intracellular signaling of pyramidal neurons in the CA1 region of the hippocampus [328, 329]. In addition, in 5-HT₇ receptor knockout mice, there is a reduced ability to induce long-term potentiation in the CA1 region of the hippocampus [327]. These findings together suggest an important role for the 5-HT₇ receptor in hippocampus-dependent functions, including learning and memory [326]. Thus, selective 5-HT₇ receptor ligands might prove therapeutically useful for the treatment of a number of neuropsychiatric disorders (Table 8.1).

8.4 FUTURE OF 5-HT RESEARCH

As has been demonstrated, the 5-HT system has been implicated in many CNS functions [1, 2], owing to the vast coverage of serotonergic projections from the raphe

nuclei throughout the CNS, the large catalog of 5-HT receptor subtypes, and their differential cellular distribution. This enormous range of actions of the 5-HT system suggests a predominantly neuromodulatory role for 5-HT in the CNS. Further highlighting the importance of the 5-HT system is the fact that the mechanism of action of most drugs currently in use for the treatment of psychiatric disorders (e.g., depression, schizophrenia, anxiety disorders, obsessive-compulsive disorder) is thought to be mediated at least in part via the 5-HT system. Thus, it is not surprising that 5-HT receptors and transporters continue to be a major focus of CNS drug discovery [2]. Advances in molecular and cellular research on individual receptor subtypes have provided new experimental tools, such as ligands with greater receptor selectivity and gene knockouts, which will allow for more sophisticated behavioral analysis of the 5-HT system. Ultimately, the integration of these advances in our understanding of the complex actions of 5-HT mediated by the various receptor subtypes will help us precisely define the overall function of 5-HT in the CNS.

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9

NEUROPHARMACOLOGY OF HISTAMINE IN BRAIN

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9.1	Organization of Histaminergic Neuronal System	301
9.1.1	Histaminergic Perikarya	301
9.1.2	Histaminergic Pathways	303
9.1.3	Afferents to Histaminergic Neurons	303
9.2	Metabolism of Histamine	305
9.2.1	Biosynthesis	305
9.2.2	Inactivation	306
9.3	Molecular Pharmacology and Localization of Histamine Receptor Subtypes	307
9.3.1	Histamine H ₁ Receptor	307
9.3.1.1	Molecular Properties	307
9.3.1.2	Signaling	309
9.3.1.3	Responses in Brain Tissues	309
9.3.1.4	Distribution	311
9.3.2	Histamine H ₂ Receptor	312
9.3.2.1	Molecular Properties and Signaling	312
9.3.2.2	Responses in Brain Tissues	312
9.3.2.3	Distribution	313
9.3.3	Histamine H ₃ Receptor	314
9.3.3.1	Molecular Properties and Signaling	314
9.3.3.2	Responses in Brain Tissues	315
9.3.3.3	Distribution	316
9.3.4	Histamine H ₄ Receptor	316
9.3.5	Interaction with NMDA Receptors	316
9.4	Histaminergic Neuron Activity and its Control	317
9.4.1	Electrophysiological Properties	317
9.4.2	Modulation of Histaminergic Neuron Activity In Vitro	317

9.4.3	Changes in Histaminergic Neuron Activity In Vivo	319
9.4.3.1	Physiological Changes	319
9.4.3.2	Pharmacological Changes	321
9.5	Physiological Roles of Histaminergic Neurons	322
9.5.1	Arousal	322
9.5.2	Cognitive Functions	324
9.5.3	Control of Pituitary Hormone Secretion	324
9.5.4	Satiation	325
9.5.5	Seizures	326
9.5.6	Nociception	326
9.6	Role of Histaminergic Neurons in Neuropsychiatric Diseases	327
9.6.1	Histamine, Schizophrenia, and Antipsychotic Drug Actions	327
9.6.2	Histamine and Alzheimer's Disease (AD)	327
9.6.3	Histamine and Parkinson's Disease	328
9.6.4	Histamine and Other Neuropsychiatric Disorders	329
9.7	Conclusion	329
	References	329

The early history of histamine started at the beginning of the twentieth century, was dominated by the works of great british pharmacologists, namely Henry Dale and W. Feldberg, and contributed to develop the common idea that this amine, ubiquitously present in a large variety of tissues (hence its name, derived from *histos*, Greek for "tissue"), was essentially playing a noxious role in the body. In agreement, the design and large therapeutic utilization of two classes of histamine receptor antagonists, the H₁ receptor antagonists by Bovet and colleagues in France [1] and the H₂ receptor antagonists by Black and colleagues in the United Kingdom [2], have allowed to alleviate in millions of patients the harmful actions of the amine when released from its two main cellular stores in peripheral tissues, that is, the mast cells and gastric enterochromaffin-like cells, respectively.

The idea that histamine may have a useful function as a neurotransmitter in brain emerged only slowly during the preceding century, essentially at the beginning of the 1970s [3], although it had been detected therein much earlier [4]. The main landmarks in this history can be summarized as follows. The development of reliable and sensitive methods to assay the amine and its synthesizing enzyme [5, 6] was instrumental in allowing to establish its cellular and subcellular localization in neurons as well as its presence in a neural pathway traveling in the medial forebrain bundle, as evidenced indirectly by lesion studies [7]. In contrast with the amine in peripheral mast cells, the turnover of the amine in cerebral neurons was found to be rapid and almost instantaneously modified by drugs such as barbiturates and reserpine [8–10]. The demonstration of depolarization-induced release and enhanced synthesis via calcium-dependent mechanisms [11–13], the elucidation of inactivating metabolic pathways [14, 15], and the characterization in brain of the H₁ and H₂ receptors by biochemical and electrophysiological approaches [16, 17] completed by the mid-1970s the "picture" of histamine as a typical monoaminergic neurotransmitter. Even more, taking into account a variety of features of the system made available at this time, it was proposed that histaminergic neurons were critically involved in the control of arousal [18].

Nevertheless it took nearly 10 years to develop reliable immunohistochemical tools which permitted to identify a tiny posterior hypothalamic area, the tuberomammillary nucleus, as the origin of the histaminergic pathways [19, 20] and, thereby, fully convince the neurobiological community of their existence: “Seeing is believing.” At approximately the same time, the third histamine receptor was identified in our laboratory, almost exclusively present in brain, where it controls the neurotransmitter release and synthesis [21], and the first selective and brain-penetrating ligands were developed [22]; these agents were used, thereafter, in hundreds of studies to modify the activity of histaminergic neurons and thereby disclose their functions.

These basic aspects, as briefly summarized above, were covered in detail in a comprehensive review [23] and will not be repeated in this chapter, which is centered on more recent studies leading to therapeutic perspectives. Other reviews on the neurobiology and pharmacology of histamine in brain have also appeared more recently [24–26].

9.1 ORGANIZATION OF HISTAMINERGIC NEURONAL SYSTEM

9.1.1 Histaminergic Perikarya

One decade after the first evidence by Garbarg et al. of an ascending histaminergic pathway obtained by lesions of the medial forebrain bundle [7], the exact localization of corresponding perikarya in the posterior hypothalamus was revealed immunohistochemically in the rat using antibodies against histamine [19] or L-histidine decarboxylase (EC 4.1.1.22, HDC), the enzyme responsible for the one-step histamine formation in the brain [27]. Data on the distribution, morphology, and connections of histamine and HDC-immunoreactive neurons were comprehensively reviewed [23, 28–31] and will be only summarized briefly here.

All known histaminergic perikarya constitute a continuous group of mainly magnocellular neurons located in the posterior hypothalamus and collectively named the tuberomammillary nucleus (Fig. 9.1). This anatomical position was observed in all mammals studied so far and in other vertebrates, including fish, reptiles, and amphibians [31], indicating that the histaminergic system is phylogenetically old and has been well preserved through the evolution. In invertebrates, histamine-immunoreactive neurons were visualized not only in the brain of molluscs [32, 33] and insects [34–36] but also in the visual system of insects [34–36], where histamine is the neurotransmitter in retinal photoreceptors, as shown by the impairment of visual behavior in HDC-deficient *Drosophila* mutants [37, 38]. The histamine-like immunoreactivity observed in photoreceptors of the turtle retina [39] and the presence of HDC-immunoreactive neurons in the guinea pig retina [40] may also indicate that histamine plays some role in the retina of vertebrates. In the rat brain, the tuberomammillary nucleus consists of about 2000 histaminergic neurons [41] and can be subdivided into medial, ventral, and diffuse subgroups extending longitudinally from the caudal end of the hypothalamus to the midportion of the third ventricle. A similar organization was described in humans, except that histaminergic neurons are more numerous (~64,000) and occupy a larger proportion of the hypothalamus [42]. Besides their large size (25–35 μm), tuberomammillary neurons

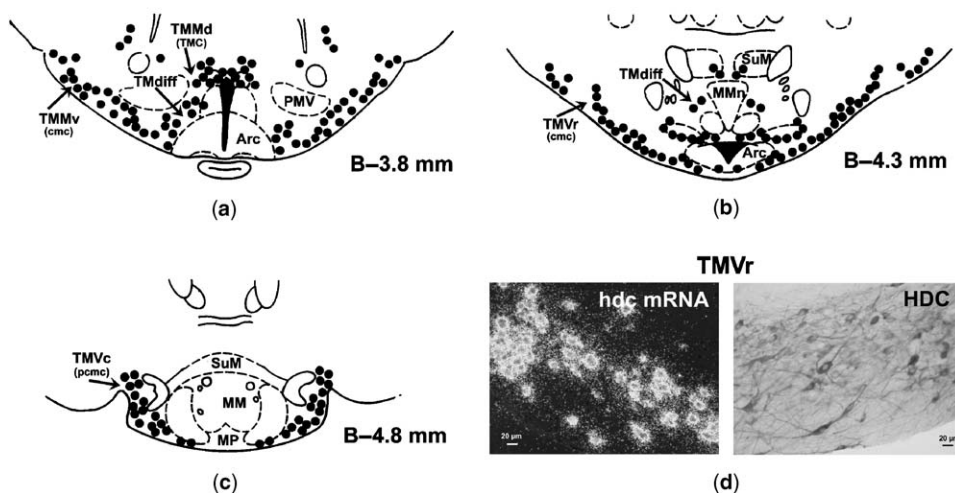


Figure 9.1 Localization of histaminergic perikarya in tuberomammillary nucleus. (a–c) Histaminergic perikarya are represented by closed circles on frontal sections at indicated levels of caudal hypothalamus. (d) Histidine decarboxylase (HDC) mRNA expression (left) and immunoreactivity (right) in ventral tuberomammillary subgroup (rostral part). Abbreviations: Arc, arcuate nucleus; cmc, caudal magnocellular nucleus; MM, medial mammillary nucleus medial part; MMn, medial mammillary nucleus median part; MP, medial mammillary nucleus posterior part; pcmc, posterior caudal magnocellular nucleus; PMV, premammillary nucleus ventral part; SuM, supramammillary nucleus; TMC, tuberal magnocellular nucleus; TMdiff, tuberomammillary nucleus diffuse part; TMMd, medial tuberomammillary subgroup dorsal part; TMMv, medial tuberomammillary subgroup ventral part; TMVc, ventral tuberomammillary subgroup caudal part; TMVr, ventral tuberomammillary subgroup rostral part.) (See color insert.)

are characterized by few thick primary dendrites, with overlapping trees, displaying few axodendritic synaptic contacts. Another characteristic feature is the close contact of dendrites or fibers with the ventral surface of the mammillary bodies and the wall of the ventricles in a way suggesting that they penetrate through the ependymal cell layer to reach the cerebrospinal fluid, perhaps to secrete or receive still unidentified messengers [43, 44]. Neurons expressing messenger ribonucleic acids (mRNAs) for histidine decarboxylase were found by in situ hybridization in the tuberomammillary nucleus but not in any other brain area [45].

The histaminergic neurons are characterized by the presence of an unusually large variety of markers for other neurotransmitter systems. Most, if not all, contain γ -aminobutyric acid (GABA) [46, 47], which is stored in subcellular structures distinct from those containing histamine [48], and glutamic acid decarboxylase, the GABA-synthesizing enzyme [49, 50]. All histaminergic neurons also contain adenosine deaminase, a cytoplasmic enzyme possibly involved in adenosine inactivation [51, 52], and many express a splice variant of choline acetyltransferase [53]. Some histaminergic neurons also express several neuropeptides such as galanin, a peptide colocalized with all other monoamines [54], (Met⁵)enkephalyl-Arg⁶Phe⁷, a product of the proenkephalin A gene, substance P, thyroliberin, or brain natriuretic peptide [55]. However, these colocalizations are observed in various proportions and display

strong species differences [47, 56]. Tuberomammillary neurons also contain monoamine oxidase B, an enzyme responsible for deamination of *tele*-methylhistamine, a major histamine metabolite in brain. Finally a subpopulation of histaminergic neurons is able to uptake and decarboxylate exogenous 5-hydroxytryptophan, a compound that they do not synthesize, however [57, 58]. Unraveling the functions of such a high number of putative cotransmitters in the same neurons remains an exciting challenge.

9.1.2 Histaminergic Pathways

In analogy with other monoaminergic neurons, histaminergic neurons constitute long and highly divergent systems projecting in a diffuse manner to many cerebral areas [28–30] (Fig. 9.2). Immunoreactive, mostly unmyelinated, varicose or nonvaricose fibers are detected in almost all cerebral regions, particularly limbic structures. It was confirmed that individual neurons project to widely divergent areas. Histaminergic fibers mainly project in an ipsilateral fashion [7], but confocal imaging of whole zebra fish or young rodent brain confirmed that commissural fiber projections containing histamine connect the two hemispheres [31]. Ultrastructural studies suggest that histamine-immunoreactive fibers make few typical synaptic contacts.

Fibers arising from the tuberomammillary nucleus constitute two ascending pathways: one laterally, via the medial forebrain bundle, and the other periventricularly. These two pathways combine in the diagonal band of Broca to project to many telencephalic areas, for example, in all areas and layers of the cerebral cortex, the most abundant projections being to the external layers. Other major areas of termination of these long ascending connections are the olfactory bulb, the hippocampus, the nucleus accumbens, the globus pallidus, the thalamus, and the amygdaloid complex. Many hypothalamic nuclei exhibit a very dense innervation, for example, the suprachiasmatic, supraoptic, arcuate, or ventromedial nuclei. A histaminergic neuronal system reminiscent of that described in rodents is present in the monkey and human brain with, for example, a dense network of fibers present in various cortical areas [59, 60] or thalamic nuclei [61], such as those belonging to the ascending visual pathways [62].

Finally, a long descending histaminergic subsystem arises also from the tuberomammillary nucleus to project to a variety of mesencephalic and brain stem structures such as the cranial nerve nuclei (e.g., the trigeminal nerve nucleus), the central gray, the colliculi, the substantia nigra, the locus ceruleus, the mesopontine tegmentum, the dorsal raphe nucleus, the cerebellum (sparse innervation), and the spinal cord.

During development, histamine is transiently present between Ed14 and Ed18 in the raphe nucleus, a neuronal system that has completely disappeared by Ed20 [63]. In the tuberomammillary nucleus, histaminergic neurons express *hdc* mRNAs from Ed14 [64] and become HDC and histamine immunoreactive from Ed16 and Ed20, respectively, whereas the development of most fibers takes place during the first two postnatal weeks [65, 66].

9.1.3 Afferents to Histaminergic Neurons

Several anterograde and retrograde tracing studies established the existence of afferent connections to the histaminergic perikarya, namely from the infralimbic

cortex, the septum–diagonal band complex, the preoptic region, the hypothalamus, and the hippocampal area (subiculum) [30, 67]. Immunoelectron microscopic studies showed that substance P and neuropeptide Y afferents make direct synaptic contacts with histaminergic neurons [68, 69]. Sleep-active GABAergic neurons in the ventrolateral preoptic nucleus (VLPO) provide a major input to the tuberomammillary nucleus [70, 71]. The contacts between these two systems are reciprocal, because the VLPO is densely innervated by histaminergic fibers [72]. Histaminergic neurons also receive very dense orexin innervation originating from the lateral hypothalamus [73]. The ultrastructure of these orexin nerve terminals confirmed direct synaptic interactions and revealed that they also contain a glutamate immunoreactivity [74, 75]. Again, the relationships between the orexin and histamine systems seem to be reciprocal, because the orexin neurons are heavily innervated by histaminergic axons [76]. The same architecture of this neuronal loop is observed in zebrafish, indicating that it is conserved in vertebrates [77]. Supporting their role in the regulation of food intake, histaminergic neurons in all subgroups of the tuberomammillary nucleus are densely innervated by nerve fiber varicosities immunoreactive for amylin- and α -melanocyte-stimulating hormone, two anorexigenic peptides [78, 79]. Electrophysiological studies provided evidence for inhibitory and excitatory synaptic control of tuberomammillary neuron activity by afferents from the diagonal band of Broca, the lateral preoptic area, and the anterior lateral hypothalamic area [80]. Projections from the brain stem to the tuberomammillary nucleus have also been demonstrated. Retrograde tracing studies combined with immunohistochemistry showed that monoaminergic inputs to the tuberomammillary nucleus originate mainly from the ventrolateral and dorsomedial medulla oblongata and from the raphe nuclei, with a lower innervation originating from the locus ceruleus, the ventral tegmental area, and the substantia nigra [81, 82].

9.2 METABOLISM OF HISTAMINE

9.2.1 Biosynthesis

Histamine biosynthesis in the brain involves two steps: transport of the precursor L-histidine (His) into the cell and its subsequent decarboxylation by HDC [23]. Saturable, energy-dependent His uptake occurs in brain slices and synaptosomes and is stimulated by their depolarization.

The human HDC gene is composed of 12 exons spanning ~24 kb. Its transcripts are alternatively spliced but only the 2.4-kb mRNA, which is predominant in human brain, encodes functional HDC [83]. The native HDC is a pyridoxal phosphate-dependent enzyme under an homodimeric form constituted of two subunits of a 54-kDa isoform and mainly found in the cytoplasm of histaminergic neurons. The primary translation product is an inactive 74-kDa isoform [84, 85], which may account for membrane-bound HDC [86, 87] and is processed at its C-terminus to give rise to the active 54-kDa enzyme [88]. The K_m of brain HDC for His is ~0.1 mM [6, 89], a value presumably close to the intraneuronal concentration of the amino acid and which accounts for the enhanced brain histamine formation induced in vivo by histidine loads. The regional distribution of brain HDC activity is consistent with data derived from immunohistochemistry, with the highest activity being found in

the hypothalamus, the lowest levels in the cerebellum, and intermediate activity in telencephalic areas [6, 23]. α -Fluoromethylhistidine (α -FMH) is a specific and irreversible inhibitor of HDC [90]. Because it easily enters the brain and efficiently depletes histamine stores in cerebral neurons, the drug is useful for investigating the amine turnover and functions therein [91, 92]. HDC knockout mice also provide a suitable model to investigate the involvement of histaminergic neurons in the regulation of fundamental functions such as sleep–wake control [93], energy homeostasis [94], learning [95], seizure development [96], motor and emotional behaviors [97, 98], and circadian rhythms [99].

In neurons, the newly synthesized histamine is transported to vesicles by the vesicular monoamine transporter 2 (VMAT2) for which it displays high affinity [100]. VMAT2 gene transcripts have been detected in the tuberomammillary nucleus [101] and VMAT2 immunoreactivity could be visualized in most histamine-containing vesicles [48].

Although histaminergic neurons constitute the major localization of HDC, at least two other types of histamine-producing cells, mast cells and microglial cells, have been reported by lesion, biochemical, and histochemical studies. Mast cells contain only a small fraction of the cerebral HDC activity but a significant fraction (up to $\sim 50\%$) of the overall brain histamine content, indicating a slow histamine turnover in this pool. Although they are rather scarce in the brain, mast cells are generally abundant in leptomeninges and also occur in the parenchyma of various brain areas, such as thalamus, where they are mainly distributed along cerebral vessels [23]. The relatively high histamine level and low HDC content found in isolated cerebral microvessels or primary cultures of cerebral microvascular endothelial cells [23, 102] may also be due to occasional histamine-containing perivascular mast cells because histamine and HDC could not be detected in endothelial cells of adult rat brain [103]. Besides neurons and mast cells, microglial cells which belong to the monocyte/macrophage lineage, contain both HDC activity and mRNAs [104]. These cells are likely to be the site of HDC activation induced by lipopolysaccharide and interleukin- 1β in cultured cells of the rat embryonic brain [105] and could be responsible for the activation of the neuronal histaminergic system in the hypothalamus [106–108].

9.2.2 Inactivation

Brain histamine is metabolized via transmethylation into *tele*-methylhistamine (*t*-MeHA) catalyzed by histamine *N*-methyltransferase (HMT, EC 2.1.1.8) [23]. In vivo inhibition of HMT leads to an increase in neuronal histamine release, confirming that this enzyme plays a critical role in histamine inactivation [109], and HMT inhibitors such as metoprine are useful drugs to investigate the histamine functions in the brain [110, 111]. HMT may be an important target to modulate histaminergic neurotransmission in neuropsychiatric disorders. Its levels are decreased in Down syndrome and increased in Pick's disease [112], and the drug tacrine, which is used in long-term palliative treatment of Alzheimer's disease, inhibits HMT even more potently than acetylcholinesterase [113]. Among the various HMT polymorphisms that have been identified in the human gene [114–116], a common C314T transition located in exon 4 resulted in decreased levels of enzyme activity [117, 118] and was associated with asthma [119] and alcoholism [120] but not with schizophrenia [121]. Although evidence for a membrane-bound HMT was reported in mouse brain [122], subcellular

fractionation studies have shown brain HMT to be mainly a soluble enzyme [23]. HMT-like immunoreactivity within the CNS was found in the cytosol of a variety of neurons and in vascular walls, whereas astrocytes were not stained [123].

How extracellular histamine is transported into these HMT-containing cells is still unclear. In contrast with other monoaminergic systems, no clear evidence for a high-affinity uptake system for histamine could be found [23]. Nevertheless, the poly-specific organic cation transporters OCT2 and OCT3 may play an important role in histamine inactivation [124]. OCT2, a “background” transporter involved in the removal of monoamine neurotransmitters, is expressed in neurons of various rat and human brain areas and mediates low-affinity transport of histamine as well as catecholamines and serotonin [125, 126]. Histamine is also a good substrate for OCT3, which was identified as the major component of the extraneuronal monoamine transport system (EMT or uptake 2) previously characterized mainly in peripheral tissues [127]. In situ hybridization revealed that OCT3 is widely distributed in different brain regions, with a prominent neuronal expression detected in cerebral cortex, hippocampus, cerebellum, and pontine nucleus [128]. Because they display a wide distribution, OCT2 and OCT3 might account at least partly for the uptake of labeled histamine reported in brain slices [129, 130].

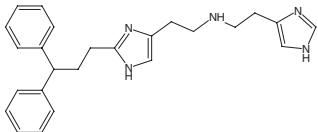
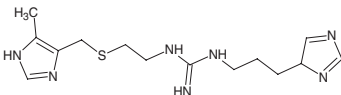
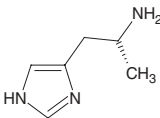
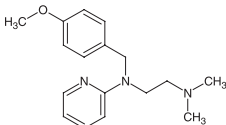
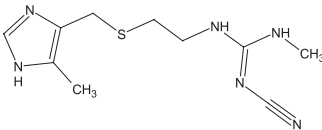
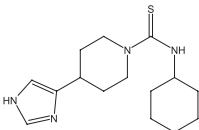
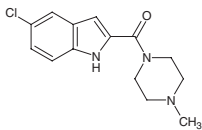
9.3 MOLECULAR PHARMACOLOGY AND LOCALIZATION OF HISTAMINE RECEPTOR SUBTYPES

In the brain, the effects of histamine are mediated by three histamine receptor subtypes (H_1 , H_2 , and H_3), which have been defined by means of functional assays followed by design of selective agonists and antagonists and, more recently, cloning of their genes [131, 132]. All three belong to the superfamily of receptors with seven transmembrane domains and coupled to guanylnucleotide-sensitive G proteins (Table 9.1). Some effects of histamine are also mediated by ionotropic receptors. Histamine directly interacts with an allosteric site of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor. At photoreceptor synapses in the insect eye, histamine activates histamine-gated chloride channels [133–135] which may also exist in the brain of vertebrates [136].

9.3.1 Histamine H_1 Receptor

9.3.1.1 Molecular Properties. The H_1 receptor was initially defined in functional assays (e.g., smooth muscle contraction) and the design of potent antagonists, the so-called antihistamines (e.g., mepyramine), most of which display prominent sedative properties. It was first cloned from cow by expression cloning [137] and subsequently from a variety of species, including man [131]. The human gene contains an intron in the 5'-flanking untranslated region, close to the translation initiation codon, but the translated region is intronless [138]. Several polymorphisms have been identified in the promoter and coding region of the H_1 receptor gene, but none of them was found to be associated with schizophrenia or response to clozapine [139–142]. Recently, the H_1 receptor was identified in the mouse as the general autoimmunity locus *Bphs*, and three polymorphisms leading to amino acid changes at positions 263, 313, and 331 in the third intracellular loop were shown to be associated with susceptibility to

TABLE 9.1 Properties of Four Histamine Receptor Subtypes^a

Property	H ₁	H ₂	H ₃	H ₄
Coding sequence	491 a.a. (b) 488 a.a. (gp) 486 a.a. (r)	358 a.a. (r) 359 a.a. (d, h, gp)	445 a.a. (h) Shorter variants (h, r, m, gp)	390 a.a. (h) 389 a.a. (gp) 391 a.a. (m, r)
Chromosome localization	3p25	5	20qTEL	18q11.2
Highest brain densities	Thalamus, cerebellum, hippocampus	Striatum, cerebral cortex, amygdala	Striatum, frontal cortex, substantia nigra	Very low density
Autoreceptor	No	No	Yes	No
Affinity for histamine	Micromolar	Micromolar	Nanomolar	Nanomolar
Characteristic agonists	<i>N</i> ^α -[2-(1 <i>H</i> -imidazol-4-yl)ethyl]histaprodifen 	Impromidine 	<i>R</i> - α -methylhistamine 	—
Characteristic antagonists	Mepyramine 	Cimetidine 	Thioperamide 	JNJ 777120 
Radioligands	[³ H]Mepyramine, [¹²⁵ I]iodobolpyramine	[³ H]Tiotidine, [¹²⁵ I]iodoaminopotentidine	[³ H] <i>R</i> - α -Methylhistamine, [¹²⁵ I]iodoproxyfan	[³ H]Histamine
Second messengers	Inositol phosphates (+), Ca ²⁺ (+), arachidonic acid (+), cAMP (potentiation)	cAMP (+), Ca ²⁺ (+)	cAMP (–), inositol phosphates (–), arachidonic acid (+), Ca ²⁺ (–)	cAMP (–)

^aAbbreviations: b, bovine; gp, guinea pig; r, rat; d, dog; h, human; m, mouse.

multiple autoimmune diseases such as experimental allergic encephalomyelitis (EAE) [143]. The marked species differences in H₁ receptor pharmacology that have been reported result from differences in binding pockets and conformational flexibility of the proteins [23, 144, 145].

9.3.1.2 Signaling. The histamine H₁ receptor produces its intracellular effects via the activation of G_{q/11} proteins [131, 146] (Fig. 9.3). Various intracellular responses were found to be mediated by stimulation of recombinant H₁ receptors or native H₁ receptors present in brain tissues and various cell systems [23, 147]. H₁ receptor activation leads to stimulation of phospholipase C β and inositol phosphate release. The subsequent mobilization of Ca²⁺ from intracellular stores followed by an influx of extracellular Ca²⁺ induces an increase in intracellular Ca²⁺ levels which was observed in transfected fibroblasts [147], neurons [148], and glial cells [149–151]. This process is presumably responsible for the activation of various Ca²⁺-dependent pathways by the recombinant or native H₁ receptor, such as potentiation of cyclic adenosine monophosphate (cAMP) accumulation, cyclic guanosine monophosphate (cGMP) accumulation, arachidonic acid release, and glycogenolysis [23, 147]. Potentiation of H₂ receptor-mediated cAMP accumulation via H₁ receptor stimulation accounts for an enhanced response to histamine in neurons coexpressing the H₁ and H₂ receptors [152].

In vasopressinergic neurons of the rat supraoptic nucleus, nitric oxide synthase activation mediated by H₁ receptors induces cGMP accumulation that results in increased interneuronal coupling [153]. Via stimulation of phospholipase C β and subsequent activation of protein kinase C α , the H₁ receptor can also signal to the nucleus and induces *c-fos* expression in CHO-transfected cells [154] or hypothalamic neurons [155, 156].

Constitutive activity of the recombinant H₁ receptor could be evidenced on several signaling pathways [157–159]. Several H₁ receptor antagonists behaved as inverse agonists, that is, reduced this constitutive activity, but the physiological relevance of the process, for example, in brain, remains doubtful [160].

9.3.1.3 Responses in Brain Tissues. The H₁ receptor mediates mainly excitatory responses in brain, leading to a depolarization and/or an increase in firing frequency in many neurons [24, 25]. A reduction of a background leakage potassium current induces excitation of cortical, striatal, and lateral geniculate relay neurons [161–163]. Activation of a tetrodotoxin-insensitive sodium current depolarizes cholinergic septal neurons [164]. The increase in intracellular Ca²⁺ levels mediated by the H₁ receptor activates a Ca²⁺-activated cation current or a Ca²⁺/Na⁺ pump that depolarizes supraoptic neurons [165] and enhances depolarizing afterpotentials, leading to an increase in firing of the same neurons [166, 167]. Stimulation of the H₁ receptor enhances the firing of serotonergic dorsal raphe neurons via activation of a mixed cation channel [168, 169]. The H₁ receptor facilitates the NMDA-mediated depolarization of cortical neurons presumably via protein kinase C (PKC) activation [170].

H₁ receptor activation also increases firing of cholinergic nucleus basalis neurons [171] and of GABAergic neurons in the substantia nigra and ventral tegmental area [172]. Hyperpolarizations or depressions of firing mediated by H₁ receptors have occasionally been observed in some neurons such as hippocampal pyramidal cells [173] or olfactory bulb neurons [174]. They mainly result from activation of a

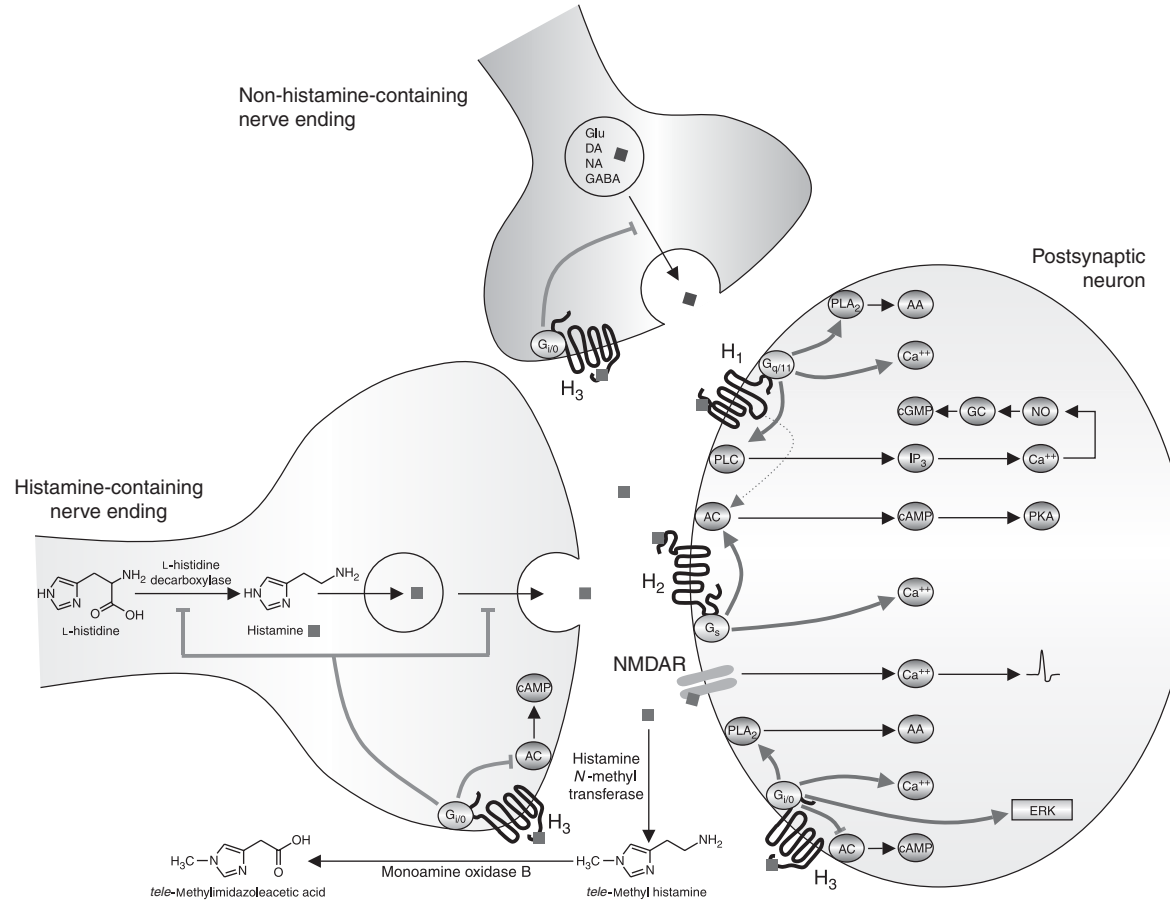


Figure 9.3 Histamine metabolism and signaling in brain. Abbreviations: AA, arachidonic acid; AC, adenylate cyclase; DA, dopamine; ERK, extracellular signal-related kinase; GABA, γ -aminobutyric acid; GC, guanylate cyclase; Glu, glutamate; IP₃, inositol-1,4,5-triphosphate; NA, noradrenaline; PKA, protein kinase A; PLA₂, phospholipase A₂; PLC, phospholipase C; NO, nitric oxide. (See color insert.)

Ca^{2+} -activated potassium conductance via release of calcium from intracellular stores [151]. The suppression of non-NMDA excitatory synaptic currents in supraoptic neurons may rather result from the production of nitric oxide [175]. H_1 receptors inhibit high-frequency (“ripple”) network oscillations in the hippocampal CA1 area via an unknown mechanism [176, 177].

In agreement with the in vitro electrophysiology, in vivo microdialysis studies show that activation of H_1 receptors in the nucleus basalis magnocellularis increases cortical acetylcholine release [178] and stimulation of H_1 receptors enhances acetylcholine release in the ventral striatum [179]. Microdialysis studies also show that activation of H_1 receptors increases noradrenaline release in the hypothalamus [180]. The increase in dopamine release induced by some H_1 receptor antagonists in the striatum presumably results from the blockade of dopamine uptake and may explain the abuse potential of such compounds [181]. In agreement, dopamine turnover remains unchanged in the forebrain from H_1 receptor knockout mice [182].

9.3.1.4 Distribution. Biochemical and localization studies of the H_1 receptor were made feasible with the design of reversible and irreversible radiolabeled probes such as [^3H]mepyramine, [^{125}I]iodobolpyramine, and [^{125}I]iodoazidophenpyramine [183, 184]. At the cellular level, although H_1 receptors are present on glial [149, 150, 185, 186] and cerebral endothelial cells [103, 187], their distribution in the brain is consistent with a predominant neuronal localization. H_1 receptor distribution in the guinea pig brain was established autoradiographically using [^3H]mepyramine or the more sensitive probe [^{125}I]iodobolpyramine [188] (Fig. 9.2) and the information complemented by in situ hybridization of the mRNA [189]. For instance, the high density of H_1 receptors in the molecular layers of cerebellum and hippocampus seems to correspond to dendrites of Purkinje and pyramidal cells, respectively, in which the mRNA is highly expressed. H_1 receptor binding sites are also abundant in guinea pig thalamus, hypothalamic nuclei (e.g., ventromedial nuclei), nucleus accumbens, amygdaloid nuclei, and frontal cortex but not in neostriatum [184]. Autoradiographic studies in the fish brain show that H_1 receptors are also localized primarily in the cerebellum and the thalamus [190]. In the human brain, binding sites are more abundant in the neostriatum than in the guinea pig [191] and, in agreement with the H_1 receptor-mediated modulation of thalamocortical functions by histamine, H_1 receptor binding sites and mRNAs are abundant in the human thalamus and prefrontal cortex [61, 192].

H_1 receptor mRNAs could be detected in rat brain from Ed14 [193], and the developmental profile of mRNAs and binding sites during the first two postnatal weeks, although not strictly parallel, is well correlated with that of histaminergic innervation [193–195]. Age-related decreases in H_1 receptors have been found in the mouse and human brain and may contribute to diminished alertness in aging [196, 197].

H_1 receptors were visualized and quantified in the primate and human brain in vivo by positron emission tomography (PET) using [^{11}C]mepyramine or [^{11}C]doxepin [198]. PET studies revealed a significant decrease in H_1 receptor binding in the brain of patients with Alzheimer’s disease [199] and of depressed or schizophrenic patients [200, 201].

Blockade of H₁ receptors in brain is presumably involved in the sedative (Section 9.5.1), pro-obesity (Section 9.5.4), and proconvulsant (Section 9.5.5) properties of many psychotropic drugs displaying high H₁ receptor affinity.

H₁ receptor knockout mice have been generated to further investigate the involvement of H₁ receptors in various neurochemical and behavioral responses. An increase in the turnover rate of serotonin was found in the mutant mice [182]. The behavioral data obtained with the knockout mice largely confirm the previous pharmacological findings and support a role of H₁ receptors in arousal and cognition [202–204], anxiety and aggressive behavior [182], nociception [205, 206], anticonvulsant action [96, 207], and regulation of food intake and body weight [208, 209].

9.3.2 Histamine H₂ Receptor

9.3.2.1 Molecular Properties and Signaling. Studies of the molecular properties of the H₂ receptor have been greatly facilitated in the 1990s by the design of its first potent and selective radioligand, [¹²⁵I]iodoaminopotentidine [210, 211], as well as by the cloning of its gene in various species, including man [131, 212, 213].

The 5'-untranslated region of the human gene contains an intron but the translated region is intronless [213, 214]. Although none of the six polymorphisms of the coding region initially reported in a U.K. population could be detected in other studies, several polymorphisms have been identified in various ethnic groups in the promoter region, and one (543G/A) in the coding region, of the H₂ receptor gene, but none were found to be associated with schizophrenia or response to clozapine [139, 140, 142, 215–217].

The recombinant or native H₂ receptor is coupled to G_s proteins and mediates activation of adenylyl cyclase with subsequent increases in cAMP formation and protein kinase A activation [23, 131, 218, 219] (Fig. 9.3). This pathway may mediate the H₂ receptor-induced ERK2 activation observed in vitro in hippocampal CA3 cells [220]. In the brain, the cAMP response mediated by H₂ receptors occurs predominantly in neurons, but it was also evidenced in astrocytes [221], epithelial cells of choroid plexus [222], and microvessel-enriched preparations [223]. Recombinant H₂ receptors also activate cAMP-independent signaling pathways and induce an increase in inositol phosphate release and intracellular Ca²⁺ concentration [224], a PKC-dependent *c-fos* activation [225], and a PKA- and PKC-independent *c-jun* activation [226]. An inhibition of arachidonate release was reported after activation of the recombinant rat receptor [227] but was not observed after activation of the human receptor [228]. Whether these various responses, which are either opposite or similar to those evoked by H₁ receptor stimulation, are activated in the brain remains unknown.

The recombinant H₂ receptor exhibits constitutive activity and several H₂ receptor antagonists behave as inverse agonists on cAMP formation [229–231]. However, constitutive activity of the native H₂ receptor in brain was not substantiated [160].

9.3.2.2 Responses in Brain Tissues. As the H₁ receptor, the H₂ receptor usually mediates excitatory responses in neurons [24, 25]. In hippocampal pyramidal neurons and granule cells of the dentate gyrus, H₂ receptor stimulation decreases a Ca²⁺-dependent K⁺ conductance through cAMP and PKA and reduces the accommodation of action potential firing, leading to an enhanced and long-lasting response

[232–235]. H_2 receptor activation depolarizes thalamic relay neurons by increasing membrane conductance, a response due to enhancement of the hyperpolarization-activated cation current I_h [162]. In striatal interneurons, H_2 receptors act synergistically with H_1 receptors to depolarize the cells via blockade of a leak potassium conductance [161]. H_2 receptor activation also increases the spontaneous firing of the alveus/oriens interneurons in the hippocampus [236], rubral neurons [237], medial vestibular nucleus neurons [238, 239], and cerebellar Purkinje and interpositus nuclear cells [240, 241]. In addition to these short-lasting effects, H_2 receptor activation also induces very long lasting increases in excitability in the CA1 region of the hippocampus, a process modulated by H_1 and NMDA receptors [173].

Depressions of firing mediated by H_2 receptor activation have also been reported in some neurons. The depressions of firing observed in several brain areas in the earlier single-unit and iontophoretic studies were most likely due to an excitation of GABAergic neurons [24]. However, in recent studies, H_2 receptor activation decreased neuronal excitability and coupling in oxytocin neurons of the supraoptic nucleus [136]. It inhibits GABAergic neurons of the thalamic perigeniculate nucleus through increases in membrane chloride conductance, a mechanism that may be involved in the transition from sleep to arousal [242]. H_2 receptor-mediated phosphorylation of voltage-gated potassium channels (Kv3.2) decreases the maximum firing frequency of hippocampal interneurons [243].

H_2 receptors that are not presynaptically located facilitate noradrenaline release from superfused guinea pig brain slices [244]. In vivo, activation of H_2 receptors enhances the release of acetylcholine in the ventral striatum [179], the medial septum–diagonal band complex [245], and the hippocampus [246, 247]. The systemic administration of zolantidine, the sole brain-penetrating H_2 receptor antagonist available [248], inhibits acetylcholine release, indicating its facilitation by endogenous histamine [246]. The decrease of ischemic release of dopamine and glutamate by H_2 receptor activation may be a contributing factor in alleviation of neuronal damage in the striatum [249].

9.3.2.3 Distribution. The H_2 receptor and/or its mRNA are detected on glial [250] and cerebral endothelial cells [103], but their distribution in the brain is consistent with a predominant neuronal localization. Autoradiographic localization of the H_2 receptor using [125 I]iodoaminopotentidine in the guinea pig [211, 251], monkey, and human brain [191, 210, 252] shows it distributed heterogeneously (Fig. 9.2). The H_2 receptor is found in most areas of the cerebral cortex, with the highest density in the external layers and the piriform and occipital cortices, the latter containing low H_1 receptor density. The caudate putamen, ventral striatal complex, and amygdaloid nuclei (bed nucleus of the stria terminalis) are among the richest brain areas. The distribution of the mRNAs is generally in agreement with that of the corresponding binding sites [192, 251, 252]. In the striatum, the absence of mRNAs in the substantia nigra and the loss of binding sites in Huntington's disease [253] indicate that H_2 receptors are expressed by intrinsic neurons. In the hippocampal formation, the relative localizations of the H_2 receptor and its gene transcripts are similar to that observed for the H_1 receptor: The gene transcripts are expressed in all pyramidal cells of Ammon's horn and in granule cells of the dentate gyrus, whereas the H_2 receptor is expressed in the molecular layers of these areas, which contain the dendritic trees of

the mRNA-containing neurons [251]. The partial overlap with the H_1 receptor may account for their synergistic interaction in cAMP accumulation. During development, H_2 receptors are transiently expressed at Ed15 together with histamine in the raphe nuclei and from Ed16 in several target areas for the histaminergic fibers [254]. mRNA levels do not change with age, except in the cerebellum, in which a significant increase has been found [197].

The brain-penetrating H_2 receptor antagonist zolantidine has been used to investigate the involvement of H_2 receptors in various neurochemical and behavioral responses [255, 256]. A rather limited number of data has also been obtained with H_2 receptor knockout mice, which tend to display a higher spontaneous locomotor activity and rearing behavior and lower susceptibility to electrically induced convulsions [257]. A number of tricyclic antidepressants are very potent inhibitors of the H_2 receptor-linked adenylylcyclase on brain membranes [258, 259] but not on intact cell preparations [260]. In addition, the idea that antidepressants derive their clinical efficacy from blockade of cerebral H_2 receptors seems unlikely because such a blockade was not observed after chronic treatments [261].

9.3.3 Histamine H_3 Receptor

9.3.3.1 Molecular Properties and Signaling. The H_3 receptor was initially detected and identified by traditional pharmacological approaches as an autoreceptor controlling histamine synthesis and release in the rat and human brain [21, 22, 262]. The recent cloning of its complementary deoxyribonucleic acids (cDNA) in various species including human and rat confirmed that the H_3 receptor is coupled to G_i/G_o proteins [263–265].

The H_3 receptor gene contains two introns in its coding sequence. These two introns, which are conserved in the human [266–268], rat [269], and mouse [270] H_3 receptor genes, are located in the second transmembrane domain and second intracellular loop, respectively. Several shorter isoforms generated by the deletion of a pseudointron in the third intracellular loop of the receptor are expressed in the brain from guinea pig [271], rat [263, 265, 269], mouse [270], and human [266, 267, 272]. The existence of these variants, which display a differential expression pattern and limited pharmacological differences, may account for the apparent H_3 receptor heterogeneity previously reported in binding or functional studies.

In addition to an isoform-related heterogeneity, H_3 receptors show a species-related heterogeneity. Although they show a high overall sequence homology, both the native [22, 262, 273, 274] and recombinant [275–278] rat and human H_3 receptors display distinct pharmacological properties, some antagonists/inverse agonists, such as thioperamide or ciproxifan, being more potent at the rat receptor, others being more potent at the human receptor. Using site-directed mutagenesis, two amino acids in the third transmembrane domain were found to be responsible for the pharmacological differences between the two species [276].

In various cell lines, activation of the recombinant H_3 receptor inhibits adenylyl cyclase [264], activates phospholipase A_2 [263], and activates the ERK signaling pathway [64, 265] (Fig. 9.3). The negative coupling of a native H_3 receptor to phospholipase C has been reported in a gastric tumor cell line [279]. However, whether any of these transductional pathways is activated by H_3 receptors in the brain remains unknown. Although a direct inhibition of adenylyl cyclase could not

be observed in various brain regions [280, 281], H_3 autoreceptors modulate histamine synthesis through the cAMP pathway [282, 283] and H_3 receptor activation inhibits dopamine D_1 receptor-mediated cAMP formation in the rat striatum [284]. Stimulation of H_3 receptors in hippocampal slices activates ERK2, but this effect does not result from a direct coupling to the ERK pathway [220].

Recombinant H_3 receptors display a high level of constitutive (or spontaneous) activity, and in fact most antagonists act as inverse agonists on various responses [263, 277, 285, 286]. Consistent with the physiological relevance of the process, constitutive activity of native H_3 receptors is detected in rodent brain [160, 263]. This constitutive activity of the native H_3 receptor is observed in all brain regions and appears to be one of the highest among G-protein-coupled receptors (GPCRs) [160]. Inverse agonists at H_3 receptors enhance histamine neuron activity by abrogating the brake triggered by constitutive activity of H_3 autoreceptors [263, 287] and are, therefore, important tools to delineate the functions of histaminergic neurons. The recombinant human H_3 receptors expressed at physiological densities also display constitutive activity, suggesting it is present in human brain [160].

9.3.3.2 Responses in Brain Tissues. After its characterization as an autoreceptor present upon histamine neurons, the H_3 receptor was shown to inhibit presynaptically the release of other monoamines in brain and peripheral tissues as well as of neuropeptides from unmyelinated C fibers. In neurons, H_3 receptors inhibit the in vitro release of various neurotransmitters, including histamine itself, noradrenaline, serotonin, dopamine, glutamate, GABA, and tachykinins [24, 131, 132, 288]. This presynaptic inhibition presumably results from a direct G-protein-mediated blockade of voltage-gated calcium channels [289–292]. In brain slices or isolated neurons, somatodendritic H_3 autoreceptors also inhibit the firing rate of histaminergic neurons by inhibiting multiple high-threshold calcium channels [293, 294]. Their tonic activation is shown by the increase in firing induced by thioperamide, a standard antagonist/inverse agonist [295]. A coupling of H_3 receptors to the neuronal Na^+/H^+ exchanger has been found in neuroblastoma cells and cardiac adrenergic neurons [296].

The inhibition mediated by H_3 autoreceptors is now well established as a major control mechanism for the activity and functions of histaminergic neurons. However, the physiological role of the H_3 receptors present on other neuronal populations remains largely unknown.

H_3 heteroreceptors inhibit dopamine synthesis [297] and release [298] but do not regulate dopamine neuron activity in vivo under basal conditions [299–301]. However, the inhibition of dopamine neuron activity by H_3 heteroreceptors may become operating after methamphetamine administration, which is known to increase histamine release [302, 303], as shown by the potentiation of methamphetamine-induced accumbal dopamine release induced by H_3 receptor antagonists/inverse agonists [304]. H_3 heteroreceptors inhibit in vitro noradrenaline release in the brain from various species, including human [244, 305, 306], but play also a minor role on noradrenaline turnover [299–301] and release in vivo [307]. H_3 heteroreceptors also inhibit serotonin release in the cortex [308] and substantia nigra [309] but do not affect serotonergic transmission under basal conditions in vivo [310]. In the striatum, dentate gyrus, and amygdala, H_3 receptor activation inhibits glutamatergic transmission in vitro [311–314], but the standard antagonist/inverse agonist

thioperamide does not increase synaptic potentials in the freely moving rat [315]. H_3 receptors inhibit GABA release in rat striatum, substantia nigra, and hypothalamus [291, 316, 317].

Acetylcholine release is also inhibited by H_3 receptors, but the latter are presumably not located on cholinergic axon terminals [318, 319]. The increase in endogenous acetylcholine release induced in vivo by H_3 receptor antagonists/inverse agonists is due to blockade of autoreceptors, increase in endogenous histamine release, and subsequent activation of H_1 or H_2 receptors [178, 179, 246, 247]. Whereas this indirect activation of the cholinergic tone is observed in several brain regions, the opposite modulation has been reported in the amygdala, the increase in acetylcholine release being induced by H_3 receptor agonists [320, 321].

9.3.3.3 Distribution. A detailed autoradiographic mapping of the rat H_3 receptor was first achieved using the highly selective agonist [3H](*R*)- α -methylhistamine [322] and more recently with the selective antagonist [^{125}I]iodoproxyfan [323] (Fig. 9.2). The comparison with the distribution of H_3 receptor mRNAs provides evidence for the presence of H_3 receptors on many neuronal perikarya, dendrites, and projections. The highest receptor densities are found in the cerebral cortex, basal ganglia, olfactory tubercles, amygdala, and tuberomammillary nucleus. Receptor densities are particularly high in the striatum, where lesions indicated that most H_3 receptors are present on projection neurons [195, 322, 324, 325]. In agreement, high densities of H_3 receptor mRNAs are also found in the striatum from rat [264, 323], guinea pig [271], and human [326]. H_3 receptors present on striatonigral neurons account for the dense binding in the substantia nigra pars reticulata [327]. H_3 receptors are also expressed in striatopallidal projection neurons [328] and account for the dense binding in the external globus pallidus in rat [323] and human [191, 326]. This expression in the external pallidum is increased in Parkinson's disease [326] and dramatically reduced in Huntington's disease [329].

H_3 receptor binding sites were not detected until day P9 [195], but H_3 receptor mRNAs could be observed as early as day E14, presumably due to the higher sensitivity of the probes [64].

H_3 receptor functions have primarily been studied with standard agonists and antagonists/inverse agonists and more recently with mice lacking H_3 receptors [330–333].

9.3.4 Histamine H_4 Receptor

Several groups recently cloned an additional histamine receptor subtype, termed H_4 , via in silico analysis of human genomic databases [334–339]. Its gene structure, coding sequence, and pharmacology are clearly related to the H_3 receptor. Only few selective ligands have so far been designed for this novel receptor and antagonists mainly display anti-inflammatory properties [340–342]. Its expression in the brain is very low, if any, and was detected only in some studies [339, 343]. Its highest level of expression is observed in hematopoietic tissues and cells (bone marrow, spleen, leucocytes).

9.3.5 Interaction with NMDA Receptors

Histamine potentiates NMDA-evoked currents in acutely isolated rat hippocampal neurons and in cultured rat hippocampal or cortical neurons [344–346].

Pharmacological studies showed that this effect could not be ascribed to activation of the metabotropic histamine receptors, since it is neither mimicked nor antagonized by several of the H_1 , H_2 , and H_3 receptor agonists or antagonists. This potentiation of NMDA receptors by histamine is slightly glycine dependent, voltage independent, and occluded by spermine. Moreover, this effect is selective for receptors containing the NR1A/NR2B NMDA receptor subunits and is likely to result from a direct potentiation of the NMDA receptor by histamine through its polyamine modulatory site [344, 345, 347].

Histamine may play a role in modulating the functions of NMDA receptors in vivo. It facilitates the NMDA-induced depolarization of projection neurons in cortical slices [170] and phase shifts the circadian clock by a direct potentiation of NMDA currents in the suprachiasmatic nucleus [348]. Furthermore histamine, presumably acting through NMDA receptors, facilitates the induction of long-term potentiation [349], causes long-lasting increases of excitability in the CA1 region of rat hippocampal slices [173], and ameliorates 7-chlorokynurenic acid-induced spatial memory deficits in rats [350].

The histamine-induced modulation of NMDA responses is higher under slightly acidic conditions [351, 352]. This may lead to enhancement of NMDA receptor-mediated neuronal excitotoxicity [353, 354], such as that observed in a rat model of Wernicke's encephalopathy [355, 356].

9.4 HISTAMINERGIC NEURON ACTIVITY AND ITS CONTROL

9.4.1 Electrophysiological Properties

Cortically projecting histaminergic neurons display typical electrophysiological properties [25, 294]. Their voltage responses, with strong inward and outward rectifications, are similar to those of other aminergic neurons but allow the unambiguous identification of histaminergic neurons in the tuberomammillary nucleus, non-histaminergic neurons in this region exhibiting quite different properties. Histamine neurons are pacemakers that display a regular spontaneous firing. Their rate of firing in vivo is higher during waking, and in vitro, they fire at rates similar to their activity during waking in vivo. Membrane conductances for Na^+ , Ca^{2+} , and K^+ are responsible for their pacemaker properties and maintain a stable and slow firing rate (below 3 Hz) in the absence of extrinsic control [357]. During spontaneous activity, the action potential is generated by a tetrodotoxin-sensitive persistent Na^+ current [358, 359] and a subthreshold voltage-dependent Ca^{2+} current [360]. Voltage- and calcium-dependent potassium currents are involved in membrane repolarization [294]. The action potential is followed by an afterhyperpolarization, which limits the firing rate. The return to the membrane resting potential is delayed by activation of a transient fast outward K^+ current of the A type [361] and facilitated by the hyperpolarization-activated I_h current [362].

9.4.2 Modulation of Histaminergic Neuron Activity In Vitro

Endogenous histamine is released by K^+ -induced depolarization of brain slices via a Ca^{2+} -, temperature- and glucose-dependent process [11–13, 363]. The

autoreceptor-regulated modulation of histamine synthesis in and release from brain neurons is well documented [23]. It was initially evidenced in brain slices or synaptosomes [21, 364, 365] after labeling the endogenous pool of histamine using the [^3H] precursor [13]. Exogenous histamine decreases the release and formation of [^3H]histamine induced by depolarization, and analysis of these responses led to the pharmacological definition of H_3 receptors. The autoregulation was found in various brain regions known to contain histamine nerve endings, suggesting that all terminals are endowed with H_3 autoreceptors.

Regulation of histamine synthesis was also observed in the posterior hypothalamus, and somatodendritic H_3 autoreceptors inhibit the firing of tuberomammillary neurons. This slowing of firing has been observed in slices, cocultures with hippocampus, and isolated histamine neurons and results from the modulation of high-voltage-activated calcium channels [232, 293, 294].

Galanin, a putative cotransmitter of a subpopulation of histaminergic neurons, inhibits histamine release only in regions known to contain efferents of this subpopulation, that is, in hypothalamus and hippocampus but not in cerebral cortex or striatum [366]. In brain slices, galanin also hyperpolarizes and decreases the firing rate of tuberomammillary neurons [367]. It is not known, however, whether these galanin receptors behave as "autoreceptors" modulating galanin release from histaminergic nerve terminals, inasmuch as the tuberomammillary nucleus receives a strong galaninergic innervation from the ventrolateral preoptic area [70, 71]. Other putative cotransmitters of histaminergic neurons failed to affect [^3H]histamine release from slices of rat cerebral cortex [368], and histamine neurons in the tuberomammillary nucleus are insensitive to adenosine [25]. However, GABAergic inhibitory postsynaptic potentials are mediated by GABA_A receptors located on histaminergic neurons [369]. The GABA_A receptors expressed among identified histamine neurons are heterogeneous and contain α_2 and α_5 subunits [370] with different expression levels of γ subunits leading to different sensitivities of histamine neurons to the transmitter [371]. To what extent these receptors play an autoinhibitory role is unclear. Histaminergic neurons contain GABA [46, 47] but the tuberomammillary nucleus also receives a dense GABAergic innervation from the VLPO [70, 71, 80]. In addition, the GABA-mediated inhibition of histamine neurons is controlled by presynaptic GABA_B and adrenergic α_2 receptors [369, 372, 373] and suppressed by dynorphin-containing neurons [295].

Orexins directly excite the histaminergic neurons in vitro [76, 374]. Most histamine neurons express mRNAs and immunoreactivity for both orexin receptors [76, 375–377], but the orexin 2 receptor seems mainly involved. In agreement, the expression of this receptor is stronger than that of the orexin 1 receptor and its absence eliminates the orexin-induced depolarization of histaminergic neurons [378].

Several other systems activate histamine neurons. Serotonin depolarizes histamine neurons and increases their firing rate by activation of $5\text{-HT}_{2\text{C}}$ receptors and a $\text{NCX1 Na}^+/\text{Ca}^{2+}$ exchanger [379, 380]. Glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors coexpressed with $\text{NCKX Na}^+/\text{Ca}^{2+}$ exchangers activate acutely isolated histamine neurons with different desensitization properties [381]. Although a purinergic transmission in the tuberomammillary nucleus has not yet been reported, adenosine triphosphate (ATP) can be coreleased with different transmitters such as GABA and excites individual histaminergic neurons through P2X receptors [382, 383]. Ghrelin, a potent orexigenic

peptide, activates cultured histamine neurons by inhibiting G-protein-coupled inward-rectifier K^+ (GIRK) channels [384], a finding in agreement with the increase in Fos expression observed in the tuberomammillary nucleus after ghrelin administration [385]. Intracellular recordings from rat hypothalamic slices indicate that morphine increases the firing of histaminergic neurons [386], and histamine release in mouse cerebral cortex is enhanced by stimulation of μ -opioid receptors [387]. Histamine release is also enhanced by stimulation of nicotinic receptors in rat hypothalamus slices [388], an effect which may result from activation of α -bungarotoxin-sensitive α_7 nicotinic receptors expressed in histamine neurons [389].

Inhibitory actions on histaminergic neurons have also been found. Besides GABA, isolated neurons with larger somata are inhibited by strychnine-sensitive glycine receptors [390]. Nociceptin inhibits the firing of histaminergic neurons by activating GIRK channels [386]. [3H]Histamine synthesis and release are inhibited in various brain regions by stimulation of not only autoreceptors but also α_2 -adrenergic receptors [391, 392], M_1 -muscarinic receptors [393], and κ -opioid receptors [394]. Since these regulations are also observed with synaptosomes, all these receptors presumably represent true presynaptic heteroreceptors. Hypothalamic histamine release differs also with sex [395], a finding which may result from the presence of estrogen receptors in most histaminergic neurons [396, 397].

9.4.3 Changes in Histaminergic Neuron Activity In Vivo

9.4.3.1 Physiological Changes. In contrast to mast cells and microglial cells, histamine neurons constitute a rapidly turning over pool in the brain [23]. Isotopic methods [398] as well as methods based upon the rates of decrease of histamine levels after inhibition of HDC [92, 399, 400] or increase in *tele*-methylhistamine (*t*-MeHA) levels after MAO inhibition [401–403] lead to a mean half-life of neuronal histamine of ~ 30 min. This value does not markedly vary among brain regions, although it is slightly higher in the hypothalamus. Both neurochemical and electrophysiological studies indicate that the activity of histaminergic neurons is maximal during arousal [23]. In rat hypothalamus, histamine levels are low whereas synthesis is high during the dark period, suggesting that neuronal activity is enhanced during the active phase [404, 405]. In mouse cerebral cortex, striatum, and hypothalamus, *t*-MeHA levels are doubled at the end of the dark phase of the cycle as compared with the beginning of the light phase [406] and are significantly enhanced during the daytime in human cerebrospinal fluid (CSF) [407]. Histamine release from the anterior hypothalamus or frontal cortex of freely moving rats or cats, evaluated by in vivo microdialysis, reaches its maximal level during periods of wakefulness [408–410]. Such state-related changes are also found in single-unit activity recordings performed in the ventrolateral posterior hypothalamus of freely moving rats or cats. Neurons identified as histaminergic neurons exhibit a circadian rhythm of their firing rate, highest during waking and falling silent during deep slow-wave or paradoxical sleep (hence referred to as “waking-on” or “rapid eye movement (REM)–off” neurons) [411–413]. In agreement, Fos expression is enhanced during periods of wakefulness in all histaminergic neurons [414].

An important determinant of this circadian rhythm of tuberomammillary histaminergic neuron activity is the GABAergic inhibitory input from the VLPO which is activated during sleep [70, 71, 80]. Muscimol, a GABA_A receptor agonist, decreases

histamine turnover in the mouse brain [415]. Histamine turnover in the brain is also rapidly reduced after administration of GABAergic sedative drugs such as barbiturates and benzodiazepines [398, 416], presumably as a result of their interaction with GABA_A receptors present on histaminergic neurons [369]. In vivo microdialysis shows that endogenous GABA as well as systemic administration of muscimol, pentobarbital, diazepam, and halothane inhibits histamine release in the rat brain [417–419]. GABA_A receptors present in the tuberomammillary nucleus play a key role in the sedative component of anesthesia. Microinjection of muscimol in the tuberomammillary nucleus produces sedation in rats and cats [420, 421], and systemic administration of muscimol, propofol, or pentobarbital decreases Fos expression in the tuberomammillary nucleus [421]. Activation of α_2 -adrenergic receptors by the general anesthetic dexmedetomidine [422] and activation of adenosine A_{2A} receptors [423] induce sleep by increasing GABA release in the tuberomammillary nucleus to inhibit histaminergic neurons.

Orexins released from neurons emanating from the lateral hypothalamus enhance histamine neuron activity. Orexin levels are not altered by circadian time [203] but their arousal effect depends on activation of histaminergic neurons [202, 424, 425]. In agreement, an altered histaminergic neurotransmission was reported in orexin receptor-2 mutated dogs, an animal model of narcolepsy [426]. Whether histaminergic neurons could represent a target for modafinil, a well-known wake-promoting drug used for the treatment of narcolepsy, is unclear. Its mechanism of action is poorly understood and its increasing effect on histamine release in vivo is indirect and is observed only after administration of high doses [427]. Fos immunoreactivity in the tuberomammillary nucleus is enhanced by administration of high [428] but not low [429] doses. Prostaglandin E₂ also induces wakefulness through activation of the histaminergic system via EP₄ receptors present on histamine neurons [430]. The very high histamine levels observed in the brain of rats with portocaval anastomosis are involved in the sleep disturbances observed in hepatic encephalopathy [431, 432]. The histamine turnover and fiber density are increased during hibernation, which may suggest that histamine neurons maintain the hibernating state or are involved in arousal from hibernation [433].

Feeding increases in vivo endogenous histamine release in the rat hypothalamus [434] and enhances Fos expression in histaminergic neurons [435]. A strong decrease in histamine neuron activity was found in Zucker fatty (*fa/fa*) rats, which have dysfunctional leptin receptors [436], as well as in obese leptin receptor-deficient *db/db* mice or leptin-deficient *ob/ob* mice [437]. That histaminergic neurons are a target for leptin in its control of feeding is also shown by the enhancement of histamine turnover and release induced in rat hypothalamus by leptin administration [437–439]. This effect of leptin may be indirect since it was not observed in two studies following intracerebroventricular administration or after transection of chorda tympani nerves, a branch of the facial nerve which mediates taste information and activates the histaminergic system [439–441]. In agreement, histamine neurons express few leptin receptors [442], and hypothalamic α -MSH (α -melanocyte stimulating hormone)-containing neurons may relay the effects of leptin on histaminergic neurons [79].

The effect of stress on histamine neuron activity depends on the stressful procedure [23]. In addition, Fos immunohistochemistry revealed a strong heterogeneity among histaminergic neurons in their response to various stress challenges [443]. Restraint stress increases histamine turnover [444] and handling stress increases

its release in vivo [445]. In contrast, food-deprived activity stress, an animal model for anorexia nervosa, decreases histamine neuron activity [446]. Changes in the metabolism and release of histamine observed in vivo after occlusion of the middle cerebral artery in rats suggest that the histaminergic activity is enhanced by cerebral ischemia [447, 448].

Ethanol decreases histamine turnover [449], and histamine levels and fiber densities are lower in the brain of genetically ethanol-sensitive rats than in ethanol-insensitive rats [450] and higher in the brain of an alcohol-preferring rat line [451].

An increase of *t*-MeHA levels with age has been reported in human CSF [452]. Changes in histamine neuron activity also occur in various neuropsychiatric diseases. *t*-MeHA levels are increased in the CSF of schizophrenic patients [453]. Histamine levels are unaffected in the brain of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated mice [454] but increased in the brain of patients with Parkinson's disease, where they are associated with a strong increase in histaminergic innervation in the substantia nigra [455, 456]. A reduced number of histamine neurons [457] and a significant decrease of HDC activity and histamine levels in the hypothalamus, hippocampus, and cortex [458, 459] have been found in Alzheimer's brains. Similar deficits have been reported in frontal cortex of patients with Down syndrome [459].

9.4.3.2 Pharmacological Changes. Whereas H_1 and H_2 receptors are apparently not involved, inhibition mediated by the H_3 autoreceptor constitutes a major regulatory mechanism for histaminergic neuron activity under physiological conditions [23]. Administration of selective H_3 receptor agonists reduces histamine turnover [22, 280] and release in vivo [460, 461]. In contrast, H_3 receptor antagonists/inverse agonists enhance histamine turnover [22, 263, 280, 462] and release in vivo [109, 463, 464], indicating that autoreceptors are tonically activated. In agreement, administration of H_3 receptor antagonists/inverse agonists also increases HDC mRNA expression [465, 466] and Fos immunoreactivity [412] in the tuberomammillary nucleus.

Agents inhibiting histamine release in vitro via stimulation of presynaptic α_2 -adrenergic receptors reduce histamine release and turnover in vivo [391, 467]. A similar inhibition is induced by activation of muscarinic heteroreceptors [393, 468, 469]. Whether these heteroreceptors are tonically activated under basal conditions remains unclear: Systemic administration of antagonists of these receptors does not enhance histamine turnover but in vivo microdialysis studies show that their local perfusion increases histamine release [467, 468, 470].

Activation of central nicotinic receptors inhibits histamine turnover [469]. Several types of serotonergic receptors are likely to modulate histamine neuron activity in vivo. 5-HT_{1A} receptor agonists inhibit histamine turnover [471], a 5-HT_{2A} receptor antagonist enhances histamine turnover [472], and a mixed 5-HT_{2C/2A} receptor antagonist decreases histamine release in vivo [473]. Perfusion of the posterior hypothalamus with dopamine D₂ receptor agonists enhances histamine release in vivo [474] and stimulation of D₂ (but not D₃) receptors by endogenous dopamine released by methamphetamine increases histamine neuron activity [302, 303].

Morphine and μ -opioid receptor agonists enhance histamine release and turnover in brain [387, 475–477]. Δ^9 -Tetrahydrocannabinol decreases histamine turnover [478], but local or systemic administration of selective cannabinoid CB₁ receptor agonists increases in vivo histamine release [479]. NMDA receptors increase in vivo release of histamine from the anterior hypothalamus [480]. Activation of NMDA and

non-NMDA receptors in the diagonal band of Broca, the lateral preoptic area, and the anterior hypothalamic area led to inhibition or enhancement of firing rates of tuberomammillary neurons [80].

9.5 PHYSIOLOGICAL ROLES OF HISTAMINERGIC NEURONS

Owing to the use of an increased number of experimental tools, for example, histamine H_3 receptor antagonists to activate histaminergic neurons [22], α -fluoromethylhistidine to block brain histamine synthesis [92], mutant mice lacking L-histidine decarboxylase [93] or the histamine H_1 receptor [182], the functional role of histaminergic neurons has been considerably clarified during recent years.

9.5.1 Arousal

Our initial proposal, in 1977 [18], that histaminergic neurons play a critical role in arousal was based upon a few experimental arguments that were put together:

1. The disposition of histaminergic neurons, at this time recently uncovered by lesion studies [7], with their highly divergent ascending projections to many telencephalic brain areas, was reminiscent of those of other aminergic systems, that is, catecholaminergic and indolaminergic systems, with an already well demonstrated role in control of sleep and arousal.
2. Intracerebroventricular administration of histamine in rabbits had been shown to induce electroencephalographic desynchronizations that were blocked by mepyramine [481, 482].
3. The well-known sedative properties of “antihistamines” could be related to blockade of histamine actions at H_1 receptors that had recently been evidenced to be present in brain [16].

This early proposal has been, since then, confirmed initially by experiments performed by Lin and Jouvet in cats [483] and Monti in rats [23], and a large variety of studies are now available to strengthen the view that the histaminergic system is one of the major neuronal systems controlling cortical activation and wakefulness (reviewed in [484]).

In agreement, ablation of these neurons, inhibition of histamine synthesis, and release or action via the H_1 receptor all decrease wakefulness and increase deep slow-wave sleep; conversely inhibition of histamine methylation or facilitation of histamine release via H_3 autoreceptor blockade increases arousal [462]. Knockout mice lacking the L-histidine decarboxylase gene ($HDC^{-/-}$ mice) show an increase in paradoxical sleep and a decrease in theta-rhythm power during waking, without, however, a deficit in daily amount of waking; nevertheless these animals introduced in a novel environment remained awake only during a few minutes whereas wild-type animals remain awake for several hours under such circumstances [93].

The arousing effect of histamine might result from H_1 and H_2 receptor-mediated depolarization of thalamic relay neurons which induces a shift of their activity from burst firing (predominating in deep sleep, during which they are poorly responsive to

sensory inputs) to single-spike activity (predominating in arousal, during which sensory information is more faithfully relayed) [485]. Arousal may also result from H_1 receptor-mediated excitation of neocortical pyramidal neurons via the same mechanism as in thalamus, that is, reduction of a background leakage potassium current [163]. Finally arousal may occur indirectly via H_1 receptor-mediated excitation of ascending cholinergic neurons within the nucleus basalis or mesopontine tegmentum [171, 483], which also induces cortical activation.

All these cellular actions of histamine, together with observations that tuberomammillary neuron firing and brain histamine turnover are maximal during wakefulness, suggest that histaminergic systems make an important contribution to the control of arousal. Histamine release measured by microdialysis in the feline anterior hypothalamus is the highest during wakefulness, intermediate during slow-wave sleep, and the lowest during paradoxical sleep, with about a maximal fivefold circadian variation [409].

The circadian changes in histaminergic neuron activity seem to be directed by two major neuronal inputs arising from the anterior/lateral hypothalamus. The first one is a slow-wave sleep-activated inhibitory GABA- and galanin-containing group of neurons arising from the VLPO area [70, 71]; in contrast, the second group of neurons releasing the neuropeptides orexins, which emanate from the lateral hypothalamus, appear to exert opposite actions since disruption of the orexin gene is associated with narcolepsy in dogs and knockout mice [73, 486]. Orexin excites the histaminergic neurons of the tuberomammillary nucleus [76]. The arousing effect of orexins may largely depend upon their activation of histaminergic neurons since the effect is absent in knockout mice deficient in the H_1 receptor [202].

The preoptic/anterior hypothalamus has been known for a long time to also play an important role in the control of sleep/wakefulness alternance because its destruction is followed by a state of prolonged insomnia; however, this state is reversed by inactivation of ventrolateral posterior hypothalamic (presumably histaminergic) neurons [487], indicating that the VLPO neurons exert a tonic inhibition upon a wakefulness center represented by the histaminergic perikarya. In turn histaminergic neurons project heavily to the anterior hypothalamus and locally applied histamine (via H_2 receptor activation) elicits wakefulness, thus witnessing the existence of a reciprocal loop between the two areas that could be involved in the alternance of sleep and wakefulness [488] according to Nauta's long-standing hypothesis (see [484] for a detailed discussion). In contrast, however, sleep-promoting GABAergic neurons in the VLPO do not appear to be affected by histamine *in vitro* [489].

Other monoaminergic, that is, catecholaminergic and indolaminergic, neurons participating in the control of sleep/wakefulness states as well as GABA/galanin VLPO neurons also receive inputs from orexin neurons which are, themselves, likely influenced by photic signals from the suprachiasmatic nucleus. In turn, neurons from the suprachiasmatic nucleus seem to be influenced in a complex manner by histaminergic inputs [490]. Hence a complex neuronal network in the hypothalamus with a series of reciprocal influences involving histaminergic neurons seems to control wakefulness.

The major part played by the H_1 receptor in these processes, confirmed in mutant mice lacking this receptor [204], accounts for the sedating effects of the first generation of "antihistamines," that is, antagonists which easily enter the brain

and are still ingredients of over-the-counter sleeping pills [491, 492]. It may also account for the sedative side effects of many antipsychotic or antidepressant drugs, a large number of which are potent H_1 receptor antagonists and were shown to block [3H]mepyramine binding to the brain receptor in rodents at subtherapeutic dosages [493, 494].

9.5.2 Cognitive Functions

The idea that activation of histaminergic neurons might improve cognitive performances is consistent with their projections to brain areas such as the prefrontal and cingulate cortices or hippocampus, their projections to cholinergic perikarya, their excitatory influences therein, and their positive role in wakefulness. The procognitive role of histaminergic neuron activation was largely established in behavioral studies in rodents using thioperamide or other, more recent, H_3 receptor inverse agonists (reviewed in [495]).

Ciproxifan, a potent and selective H_3 receptor inverse agonist, which strongly enhances histamine turnover in brain, improved attentional performances in the rat five-choice test under conditions similar to those of drugs enhancing cholinergic transmissions [462]. The same drug, as well as thioperamide and several other compounds of the same pharmacological class, also exerted proattentional activity in a five-trial acquisition test performed in spontaneously hypertensive rat pups, often considered as a model for attention deficits and impulsivity in attention-deficit hyperactive disorder (ADHD) patients [496].

Various H_3 antagonists facilitate various forms of learning: memory consolidation, spatial orientation, short-term memory, and working memory [495]. For instance, they improve short-term social memory in rats [497], reverse the scopolamine- or senescence-induced learning deficit in a passive avoidance test in mice [498], and facilitate retention in a footshock avoidance test in mice [499].

Generally H_3 agonists exert opposite effects and the effects of H_3 antagonists are reversed by H_1 antagonists, which suggests that the latter are attributable to enhanced histamine release. In agreement, H_3 receptor knockout mice display enhanced spatial learning and memory [333].

In contrast to the large body of experiments indicating a “procognitive” role of tuberomammillary neurons, Huston and co-workers have repeatedly shown that excitotoxic lesions, aimed at these neuron ablations, improves learning in a variety of tests (e.g., [500]). The discrepancy with data derived from pharmacological approaches might be due to the difficulty to achieve selective histamine neuron ablation [501]. Also a more confusing picture emerges from studies in which the drugs were applied intracerebrally, a procedure which is likely to lead to artifacts [502]. Nevertheless, HDC knockout mice show deficient nonreinforced episodic object memory but improved negatively reinforced water-maze performance [95].

9.5.3 Control of Pituitary Hormone Secretion

Histamine affects secretion of several pituitary hormones [23, 503]. Magnocellular neurons of the supraoptic and paraventricular nuclei are typically excited, an essentially H_1 receptor-mediated response resulting in enhanced blood levels of vasopressin and oxytocin. Histaminergic neurons are activated during dehydration,

parturition, and lactation, and histamine release onto magnocellular neurons participates in the control of these physiological processes by the neurohypophysial hormones [504–506].

Histaminergic neurons may also participate in the hormonal responses to stress. In agreement they are activated during various forms of stress and heavily project to hypothalamic or limbic brain areas (e.g., amygdala or bed nucleus of the stria terminalis) involved in these responses. Various pharmacological studies have shown the participation of endogenous histamine via H_1 and H_2 receptor stimulation in the adrenocorticotrophic hormone (ACTH), corticosterone, prolactin, or renin responses to stressful stimuli like restraint, endotoxin, or dehydration [507, 508]. Furthermore, it seems that the activation of various subpopulations of histaminergic neurons within the tuberomammillary nucleus varies according to the nature of stressful stimuli [443, 509].

A high proportion of histaminergic neurons contain estrogen receptors, project to luteinizing hormone releasing hormone (LHRH) neurons in preoptic and infundibular regions, and might constitute, via H_1 receptor stimulation, an important relay in the estradiol-induced preovulatory LH surge [396]. Both estrogen receptors α and β were characterized in large (presumably histaminergic) neurons of the human brain and their role in the control of these neurons activity suggested [397].

9.5.4 Satiation

A satiating role of endogenous histamine is strongly suggested by several observations, but this view is not in agreement with all experimental data.

For instance, weight gain is often experienced by patients receiving first-generation H_1 antihistamines crossing the blood–brain barrier as well as antipsychotics or antidepressants displaying potent H_1 receptor antagonist properties. This eventually results in an increased risk of developing a “metabolic syndrome” in patients chronically treated with such psychotropic agents (e.g., second-generation antipsychotics) [494, 510]. Central infusion of histamine reduces fat accumulation in leptin-resistant obese mice [511]. These effects could reflect the inhibitory role of endogenous histamine on food intake mediated by the H_1 receptor, namely on the ventromedial nucleus [512].

Histamine neurons projecting to the hypothalamus may be responsible for the food intake suppression induced by the fat cell–produced hormone, leptin. In agreement, intracerebroventricular administration of leptin increases hypothalamic histamine release [438], whereas histamine depletion by α -fluoromethylhistidine treatment attenuates leptin-induced feeding inhibition [437]. Aged mice with gene disruption of either the H_1 receptor [209] or the histidine decarboxylase gene [94] display adiposity; also both mice display hyperleptinemia, which suggests the existence of a regulatory loop between hypothalamic histamine neurons and leptin-producing cells, the nature of which remains elusive.

Following the initial observation that the prototypical H_3 receptor antagonist/inverse agonist thioperamide decreases food intake in rats [512], studies using various compounds belonging to this drug class have confirmed that increased brain histamine release in rodents is associated with anorectic and antiobesity effects (reviewed in [513]). For instance, treatment of mice stabilized on a high-fat diet with A-331440, a potent non-imidazole H_3 receptor antagonist, decreased weight

comparably to dexfenfluramine, reduced body fat, and normalized insulin resistance test [514].

Nevertheless not all H_3 receptor inverse agonists elicit such effects (S. Krief, personal communication); thioperamide-induced appetite suppression was attributed to taste aversion to this compound [515], and in a mouse model of H_3 receptor disruption, paradoxical increases in body weight, food intake, and adiposity together with reductions in energy expenditure have been reported [331].

9.5.5 Seizures

The anticonvulsant properties of endogenous histamine were initially suggested from the occurrence of seizures in epileptic patients, particularly children, following administration of high doses of H_1 receptor antagonists crossing the blood–brain barrier, even those devoid of anticholinergic activity [491].

The role of histaminergic neurons in preventing seizures, or even the development of epileptogenesis, presumably, in most cases, via H_1 receptor activation, has been shown in several rodent models of epilepsy [516, 517]. In agreement, drug-induced changes in histamine synthesis, release, or metabolism confirmed the role of the endogenous amine acting via the H_1 receptor in preventing seizure activity elicited in rodents by pentetrazole, transcranial electrical stimulation, or amygdaloid kindling. Consistently, H_3 antagonists inhibit amygdaloid kindled seizures, an effect prevented by H_1 antagonists, which suggests the involvement of endogenous histamine [518]. Even more, studies in histidine decarboxylase- and H_1 receptor-deficient mice indicated that endogenous histamine plays a crucial role not only in amygdaloid kindled seizures but also in the development of amygdaloid kindling [207]. Acquired amygdaloid kindling susceptibility appears associated with reduced histamine synthesis in limbic brain areas [519].

In addition, a protective role of endogenous histamine was convincingly shown in the development of pentetrazole-induced kindling in rats or mice [96, 520] as well as in the development of seizures in EL mice, a model for hereditary temporal lobe epilepsy [521].

In another epilepsy model, kainic acid-induced limbic seizures in the rat, upregulation of the H_1 receptor mRNA is detected in striatum and dentate gyrus, consistent with a regulatory role of this receptor in seizure activity [194].

Taken together all these studies suggest that enhancing brain histamine release via H_3 receptor blockade should represent a novel therapeutic approach for several epilepsies.

9.5.6 Nociception

The antinociceptive effects of histidine loads, H_3 receptor antagonists, and histamine *N*-methyltransferase inhibitors as well as opposite effects of histamine synthesis inhibitors or H_3 agonists support the idea that brain histamine inhibits nociceptive responses such as the mouse hot-plate jump [522]. In contrast, peripherally acting H_3 receptor agonists prevent nociceptive responses such as mouse abdominal constriction by inhibiting sensory C-fiber activity [523]. Activation of spinal H_3 receptors was shown to inhibit mechanical but not thermal nociceptive responses [524].

9.6 ROLE OF HISTAMINERGIC NEURONS IN NEUROPSYCHIATRIC DISEASES

Various approaches tend to establish the implication of histaminergic neurons in neuropsychiatric diseases, although the evidence remains largely indirect due to the poor predictability value of most animal models and the paucity of drugs affecting histaminergic transmission that were tried in these human diseases so far.

9.6.1 Histamine, Schizophrenia, and Antipsychotic Drug Actions

Overdose of a variety of classical H₁ antagonists was repeatedly reported to result in toxic psychoses with hallucinations resembling schizophrenia, and the hallucinogenic potential of these drugs has even led to abuse [491].

On the other hand, methamphetamine, a drug with hallucinogenic potential and to which schizophrenic patients seem hyperresponsive, releases histamine in rodent brain areas, an indirect effect mediated by stimulation of D₂ and not D₃ dopamine receptors [302, 303, 472]. Even more, endogenous dopamine appears to exert a tonic stimulation of histamine neurons since typical neuroleptics (e.g., haloperidol) decrease their activity. In contrast, atypical neuroleptics (e.g., clozapine) enhance histamine turnover, an effect related to 5-HT₂ receptor blockade and possibly underlying their procognitive properties [472]. The locomotor activation elicited in rodents by amphetamine and other dopaminergic agonists is attenuated by H₃ receptor blockade [525]. Repeated amphetamine administration to rodents which results in behavioral sensitization to dopamine agonists, a cardinal feature of schizophrenia, is accompanied by enhanced histamine release, which presumably reflects an enhanced tonic dopaminergic influence on histaminergic neurons [302, 303]. In another animal model of psychosis, the locomotor stimulation induced in rodents by the NMDA antagonist dizocilpine, a “psychotogenic” agent, is also partially blocked by H₃ receptor inverse agonists (R. Faucard, unpublished observation). In two other models of schizophrenia in which sensorimotor gating deficits, which are considered as cardinal signs of the disease, were monitored in rodents, for example, prepulse inhibition of a startle response or the auditory-evoked N40 electroencephalographic wave, a normalization was reported upon administration of these drugs [526]. In one comprehensive study, an enhanced level of *t*-MeHA, the major histamine metabolite, was detected in the CSF of schizophrenic patients, either treated or nontreated by neuroleptics [453]. Ciproxifan, an H₃ receptor antagonist/inverse agonist, potentiates neurochemical and behavioral effects of haloperidol in the rat [328].

In several open studies famotidine, an H₂ antagonist, was found to improve schizophrenic patients, a finding which remains to be confirmed in control studies. A previous claim of association between polymorphisms of the H₂ receptor gene and schizophrenia could not be confirmed [216].

These various observations, although not easily forming a coherent picture, suggest that histaminergic neuron activity is enhanced in schizophrenic patients and that blockade of H₂ and/or H₃ receptors might be useful in schizophrenia treatment.

9.6.2 Histamine and Alzheimer's Disease (AD)

In spite of some initial inconsistencies, several neuropathological studies have clearly documented a deficit in histaminergic neurotransmission in Alzheimer's disease (AD).

Numerous neurofibrillary tangles are found in the tuberomammillary nucleus of the AD brain, but most of them are extracellularly located and few are encountered within the histamine-immunoreactive neurons: They were suggested to correspond to remnants of degenerated histaminergic neurons [42, 457]. In agreement, in some (e.g., frontal or temporal cortex, hippocampus) but not all cortical areas of AD brains, there is a decrease of histamine and histidine decarboxylase levels that may reach up to ~50% [458, 459], although these indices of histaminergic fiber degeneration may not reach corresponding indices for cholinergic neurons. Furthermore, there is some evidence that the activity of remaining histaminergic neurons is reduced: Cell and Golgi apparatus size [397] and the expression of the *hdc* gene (S. Trottier and D. F. Swaab, personal communication) are reduced in neurons of the tuberomammillary nucleus.

In addition histamine H₁ receptor abundance, assessed by PET, is significantly reduced in AD patients, particularly in frontal and temporal brain areas [199].

Decreased histaminergic input may affect cholinergic neuron activity in nucleus basalis [171] and acetylcholine release in cortical areas. Taking into account an additional direct positive influence of histamine on attention and memory, this indicates that the histaminergic deficit may participate in the cognitive impairments of AD; correlatively, enhancing histaminergic neurotransmission (e.g., by H₃ receptor antagonists, which were found to compensate cognitive deficits elicited by blockade of cholinergic transmission in rodents) may constitute a novel, symptomatic treatment of the disease. Interestingly, the drug tacrine was even more potent in inhibiting histamine *N*-methyltransferase, the main histamine-metabolizing enzyme, than acetylcholinesterase [113].

In Down syndrome, in which neuropathological changes are similar to those seen in AD, a deficit in histaminergic neurons was also documented [459].

9.6.3 Histamine and Parkinson's Disease

Histamine neurons, which project abundantly in the striatal complex, do not appear to degenerate during Parkinson's disease as judged from levels of L-histidine decarboxylase activity [527] and histamine [455] in postmortem samples of striatum. In contrast, the density of histaminergic fibers in substantia nigra pars compacta and reticulata seems increased in the disease, a change possibly reflecting a compensatory process to dopaminergic neuron depopulation [456].

The interactions between nigrostriatal dopaminergic and histaminergic neurons seem rather complex. Dopaminergic neurons express presynaptic H₃ receptor exerting an inhibitory action on the amine release [298]. Ciproxifan, a potent inverse agonist at the H₃ receptor, strongly potentiated the effect of methamphetamine on proenkephalin expression in rat striatum but depressed its effect on prodynorphin and substance P expression, thus indicating a differential modulation of the activity of striatonigral and striatopallidal neurons [528]. This suggests that the modulatory role of H₃ receptors upon the nigrostriatal complex might find applications in the treatment of Parkinson's disease. In agreement, thioperamide [529] was found to potentiate the levopoda-induced turning behavior of hemiparkinsonian rats.

9.6.4 Histamine and Other Neuropsychiatric Disorders

Anxiety might be increased by endogenous histamine acting at the H₁ receptor. In agreement, H₁ receptor knockout mice display significantly less anxiety in the elevated maze test [182]. However, the utility of H₁ receptor antagonists in anxiety disorders is not established. In addition, H₃ receptor knockout mice showed reduced measures of anxiety [333].

ADHD may benefit from enhanced histamine release as suggested by the therapeutic effect of amphetamine in children and the attention-enhancing effects of an H₃ receptor antagonist in the rat [462]. The diminished cognitive performances of spontaneously hypertensive rat pups, a model for ADHD, are improved by H₃ receptor antagonists, presumably as a result of their histamine-releasing effects [496].

Antidepressant-like effects in the mouse forced swim test result from enhanced histamine release and H₁ receptor activation [530].

9.7 CONCLUSION

This chapter testifies how our knowledge of the molecular neurobiology of cerebral histaminergic systems and their implications in physiological functions (e.g., arousal, cognition, or control of food intake) has progressed during the last years. This appears as the result of the development of reliable research tools such as selective ligands for the various receptor subtypes or genetically modified mice. In contrast, little is known, so far, about the possible implications of histaminergic neurons in neuropsychiatric diseases and the therapeutic utility of psychotropic drugs affecting their activity. H₃ receptor antagonists (or inverse agonists) which markedly enhance brain histamine release are currently undergoing clinical trials in several CNS disorders. It seems likely that such studies will teach us a lot about the role of histamine in the human brain.

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IONOTROPIC GLUTAMATE RECEPTORS

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10.1	Background/Receptor Classification	365
10.1.1	Glutamate Synthesis and Storage	366
10.1.2	Ionotropic Glutamate Receptor Subtypes	366
10.1.3	NMDA Receptors	367
10.1.4	AMPA Receptors	370
10.1.5	Kainate Receptors	371
10.2	Orthosteric Pharmacological Agents	373
10.2.1	NMDA Receptor Orthosteric Agonists	373
10.2.2	NMDA Receptor Orthosteric Antagonists	373
10.2.3	AMPA Receptor Orthosteric Agonists	376
10.2.4	AMPA Receptor Orthosteric Antagonists	377
10.2.5	Kainate Receptor Orthosteric Agonists	378
10.2.6	Kainate Receptor Orthosteric Antagonists	381
10.3	Allosteric Potentiators and Antagonists	385
10.3.1	NMDA Receptor Allosteric Potentiators	385
10.3.2	NMDA Receptor Allosteric Antagonists	386
10.3.3	AMPA Receptor Allosteric Potentiators	390
10.3.4	AMPA Receptor Allosteric Antagonists	395
10.3.5	Kainate Receptor Allosteric Potentiators	396
10.3.6	Kainate Receptor Allosteric Antagonists	397
10.4	Future Perspectives	397
	References	398

10.1 BACKGROUND/RECEPTOR CLASSIFICATION

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. There have been significant advances in our understanding of the

molecular physiology of the proteins activated by glutamate (metabotropic and ionotropic glutamate receptors) as well as the pharmacology of agents that interact with these proteins. The original classification of ionotropic glutamate receptors was based on pharmacological selectivity and included receptor subtypes preferentially responsive to *N*-methyl-D-aspartate (NMDA), kainic acid (KA), and quisqualic acid [1–3]. Subsequent studies showed that quisqualate also activated inositol phospholipid hydrolysis in hippocampal slices, cultures of cerebellar granule cells and striatal cells, providing some of the first evidence for the existence of metabotropic glutamate receptors [4, 5]. In addition, the discovery that α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was a more selective agonist for quisqualate receptors than quisqualate itself [6] prompted a change in nomenclature of non-NMDA receptors to AMPA and kainate receptors [7]. Ensuing molecular biological studies have confirmed the fitness of this classification scheme through identification of separate genes encoding distinct subunits for NMDA, AMPA, and kainate receptors (for review, see [8]).

10.1.1 Glutamate Synthesis and Storage

It is now understood that interactions between neurons and glia play an important role in the storage and synthesis of glutamate (reviewed by [9]). Following release, glutamate is removed from the synaptic cleft and extracellular space by cellular uptake. Both neurons (presynaptic and postsynaptic) and glial cells express functional glutamate transporter proteins that use electrochemical gradients to drive glutamate uptake (reviewed in [10]). In nerve terminals, glutamate is transported into synaptic vesicles via a vesicular glutamate transporter (VGLUT1 and VGLUT2) [11]. As such, glutamate may become a metabolic substrate or be used as a transmitter by exocytosis. In astrocytes, it appears that following uptake, glutamate can be converted to glutamine by glutamine synthetase and/or converted to α -ketoglutarate (by glutamate dehydrogenase or aspartate aminotransferase) [12]. Glutamine and α -ketoglutarate can be released from astrocytes and be taken up by neurons. Neuronal glutamine can be converted to glutamate by the adenosine triphosphate (ATP)-dependent enzyme glutaminase and α -ketoglutarate to glutamate by transamination.

10.1.2 Ionotropic Glutamate Receptor Subtypes

Within each of the three ionotropic glutamate receptor groups there are individual subunits; seven for NMDA, four for AMPA receptors, and five for KA receptors (Fig. 10.1). For each receptor type, it is likely that four subunits assemble to form an ion channel, that, dependent upon subunit composition, is permeable to Na^+ and Ca^{2+} ions. In addition to this tetrameric structure, NMDA, AMPA, and KA receptors share similar subunit transmembrane topology with an extracellular amino terminus (S1 region), a first transmembrane region, a pore forming region that consists of a loop that enters the plasma membrane and returns to the cytoplasm, a transmembrane region that forms a large extracellular loop (S2), and a third transmembrane region that enters the cell and forms a carboxy terminus (Fig. 10.2). Based upon crystal structure information, ligand binding for glutamate (and other orthosteric agonists) appears to be between the S1 and S2 regions [13, 14].

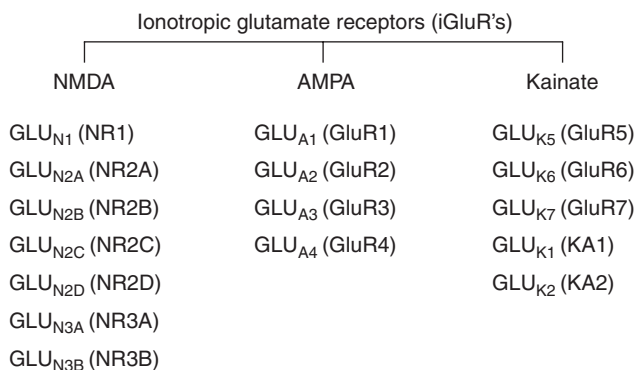


Figure 10.1 Receptor classification.

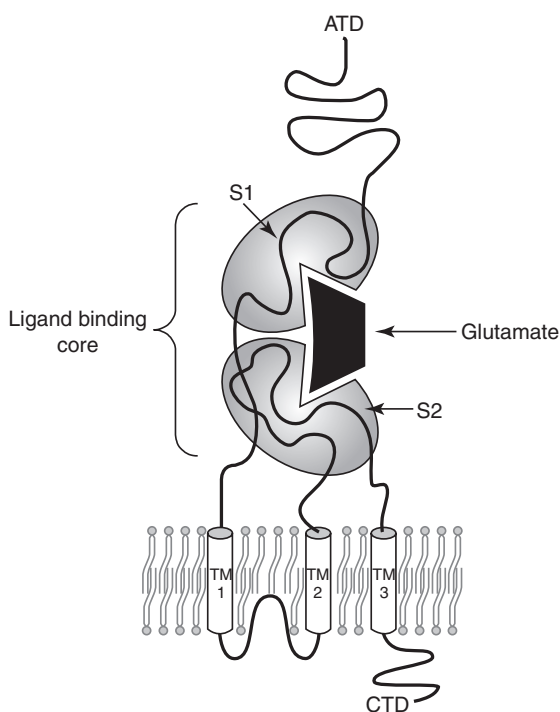


Figure 10.2 Structure of generic ionotropic glutamate receptor.

10.1.3 NMDA Receptors

N-Methyl-D-aspartic acid (Fig. 10.3) was one of the early compounds synthesized to study the structure–activity relationships of the natural excitatory acidic amino acids, L-glutamate and L-aspartate, and proved to be considerably more potent than the endogenous substances [15, 16]. Interestingly, unlike these two natural amino acids which showed only limited stereoselectivity, NMDA was far more potent as an excitant than *N*-Methyl-L-aspartic acid [17]. Over the next 10–15 years, NMDA gave

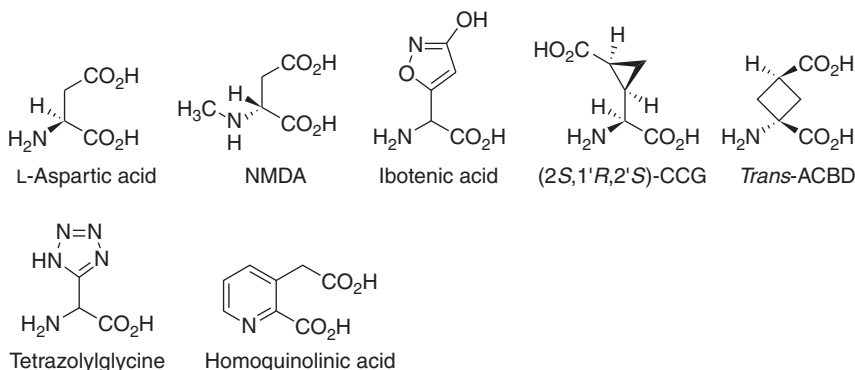


Figure 10.3 NMDA receptor orthosteric agonists.

its name to a subtype of receptor that was sensitive to a series of structurally distinct antagonists, discovered with some degree of serendipity and a lot of hard grind by chemists and biologists alike. These early compounds included magnesium ions [18], α,ϵ -diaminopimelate (DAP) [19], α -aminoadipate (Fig. 10.3) [20, 21], and 3-amino-1-hydroxypyrrolidin-2-one (HA-966; see Fig. 10.9 below) [19, 22], each of which antagonized NMDA-induced excitations but were far less effective against quisqualate- or kainate-induced excitations of spinal neurones. Pharmacological division between quisqualate (now AMPA) and kainate receptor subtypes resulted from the use of glutamic acid diethylester [2, 6] and later compounds as described in other sections of this chapter. The structural diversity of the early NMDA antagonists was a forerunner for the elucidation of multiple sites on the NMDA receptor complex that will form the main part of this section.

Alongside these pharmacological studies were parallel attempts to show the importance of NMDA and the other glutamate receptor subtypes in synaptic transmission [1, 19, 20, 23]. Better antagonists were needed. For the NMDA receptor, this soon occurred with the marked improvement in selectivity and potency of (*R*)-2-amino-5-phosphonopentanoate (Fig. 10.3) [24], the ω -phosphonate analog of D- α -aminoadipate. Studies with D-AP5, as it is commonly called, led to demonstrations of the importance of the NMDA receptor in synaptic plasticity [25], in neuronal cell death [26], and in convulsive states [27] and a general interest in their role in neurological conditions (see [28]). Indeed, NMDA antagonists have been shown to have potential clinical value in such apparently diverse neurological conditions as stroke [29], epilepsy [30], and pain [31]. In such conditions overactivity of the glutamatergic system leads to membrane depolarization, relief of the voltage-dependent magnesium block of the NMDA receptor channel (see below), calcium entry, and subsequent cascade of intracellular events leading to specific symptomatology.

A subsequent finding that the psychoactive drugs ketamine and phencyclidine (see Fig. 10.10 below) were also NMDA antagonists [32] initiated increased interest in the role of these receptors in psychiatric diseases [33]. In particular, the psychoactive properties of phencyclidine and other allosteric antagonists of NMDA [34] underpinned the hypoglutamatergic hypothesis of schizophrenia (see [35]). The discovery that glycine was a necessary coagonist at the NMDA receptor [36, 37] and that polyamines influence NMDA receptor function [38] added to the increasing diversity and to the potential number of molecular sites to design pharmacological agents.

Before considering this pharmacology and its subsequent development in detail, we need to know something of the basic molecular biology and structure of the NMDA receptor. It is now generally accepted that the NMDA receptor is a tetramer of subunits forming a receptor channel complex. Cloning and expression of NMDA receptor units have elucidated seven genes coding for seven subunits, namely GLU_{N1} , GLU_{N2A} , GLU_{N2B} , GLU_{N2C} , GLU_{N2D} , GLU_{N3A} , and GLU_{N3B} (alternatively referred to as NR1, NR2A, etc.) [8, 39]. There are multiple splice variants of GLU_{N1} and distinct anatomical developmental expression of the GLU_{N2} and GLU_{N3} subunits. Each receptor consists of a large extracellular N-terminus, three transmembrane segments, a pore-lining intramembrane loop between the first and second transmembrane segments, a large extracellular loop between the second and third transmembrane segments, and a relatively small intracellular C-terminus. The two large extracellular domains interact via the S1 and S2 segments to form the agonist binding site. Numerous studies have attempted to define the subunit structure of native and recombinant receptors. It is the general consensus that two GLU_{N1} subunits associate with either two GLU_{N2} or two GLU_{N3} subunits or with one of each to form the native receptor; the two GLU_{N2} may be different gene products, but the combinations that exist in native NMDA receptor complexes have not yet been fully defined. In the most common form of the NMDA receptor, glutamate binds to the GLU_{N2} subunits and the coagonist, glycine, binds to the GLU_{N1} subunits. Recently X-ray crystallographic studies on GLU_{N2A} have confirmed that the GLU_{N1} – GLU_{N2A} heterodimer is the functional unit in the tetrameric NMDA receptor [40]. One of the goals of researchers in the NMDA field is to have compounds showing selectivity for the GLU_{N2} subtypes, which will aid in elucidating the function of the various potential associations. It seems likely that the combination of GLU_{N1} and GLU_{N3} subunits is not sensitive to glutamate or NMDA but rather results in an excitatory glycine receptor [41] and, in other heteromeric combinations, expression of GLU_{N3} reduces NMDA-induced currents relative to GLU_{N1} / GLU_{N2} combinations.

In addition, nonhomologous asparagine residues on the GLU_{N1} and GLU_{N2} subunits produces a narrow constriction in the heteromeric ion channel that allows Ca^{2+} but not Mg^{2+} to permeate [42]. This restricted Mg^{2+} access is important for generating the voltage-dependent block that is relieved upon depolarization.

NMDA receptors have a widespread distribution in the central nervous system (for reviews see [43, 44]). However, *in situ* hybridization studies show that some of the GLU_{N2} subunits have a more restricted localization [45, 46]. For example the GLU_{N2C} subunit is expressed almost exclusively in the cerebellum and GLU_{N2D} is found throughout the diencephalon, midbrain, and glomerular layer of the olfactory bulb [45]. Both GLU_{N2A} and GLU_{N2B} have a distinct but widespread distribution in the central nervous system. For instance, GLU_{N2A} but not GLU_{N2B} has a high expression in the cerebellum whereas GLU_{N2B} but not GLU_{N2A} is highly expressed in the septum [45]. Four pharmacologically distinct NMDA receptor subtypes have been identified in the central nervous system and these have been shown to differ in their GLU_{N2} subunit composition [45, 47–49].

There is a change in GLU_{N2} subunit expression during development with GLU_{N2A} being abundant in adults whereas GLU_{N2B} is abundant in the forebrain throughout development to the adult stage. Interestingly, after postnatal days 10–11 there is a switch in cerebellar granule cell expression of GLU_{N2B} messenger ribonucleic acid (mRNA) for GLU_{N2C} and GLU_{N2A} [46, 50]. The GLU_{N2D} subunit

is abundant in the spinal cord and brain stem throughout development but not in the adult (see review by Watanabe [51]).

In adults, *in situ* hybridization studies demonstrated that the GLU_{N3B} subunit is expressed mainly in the ventral horn of the spinal cord (particularly in motor neurons) and in the facial and trigeminal nuclei of the brain stem [41] whereas the GLU_{N3A} subunit is more widely distributed [52, 53].

10.1.4 AMPA Receptors

The AMPA receptor family includes four different genes termed $\text{GLU}_{\text{A1-4}}$ (GluR1–4 or GluRA–D) that encode proteins of approximately 900 amino acids and share approximately 70% amino acid identity [43, 54]. Structurally, these proteins contain a large extracellular N-terminus domain and four hydrophobic domains labeled TM1–TM4 (Fig. 10.2). In reality, evidence suggests that AMPA receptor subunits have only three transmembrane domains (TM1, TM3, and TM4 in the original nomenclature) and a reentrant loop (TM2) on the cytoplasmic side. This reentrant loop is believed to line the pore of the ion channel, and so is sometimes referred to as the P domain. This topology indicates that both the large N-terminus and the region between TM3 and TM4 are extracellular. The region of the N-terminus, immediately adjacent to TM1 (designated S1), and the region between M3 and M4 (designated S2) have been crystallized and shown to form the ligand binding core (LBC) having an upper and lower domain that gives rise to a “clamshell” configuration with the orthosteric recognition site being localized within the mouth of the clamshell (Fig. 10.2) [13, 55].

Functional AMPA receptors are proposed to be tetramers that can be generated by the assembly of one or more of the protein subunits $\text{GLU}_{\text{A1-4}}$, yielding homomeric or heteromeric receptors [56]. $\text{GLU}_{\text{A1-4}}$ subunits can be modified posttranscriptionally to further increase the diversity of AMPA receptors. RNA editing occurs at two different positions within the AMPA receptor genes. A glutamine residue (Q; CGA) in the pore region of GLU_{A2} is edited to give an arginine (R; CGG). In fully developed adult animals, virtually all of the GLU_{A2} subunit is edited at this site. This edited residue significantly reduces calcium permeability, decreases single-channel conductance, and reduces rectification of the AMPA receptor [57, 58]. A second site in the extracellular domain between TM2 and TM3 is edited from an arginine (R; AGA) to a glycine (G; GGA) in GLU_{A2} , GLU_{A3} , and GLU_{A4} . This edited residue can alter the time course of recovery from desensitization [59].

Additional complexity among AMPA receptors results from alternative splicing in the extracellular S2 region in $\text{GLU}_{\text{A1-4}}$. This region can contain one of two different exons, referred to as flip (i) and flop (o) [60]. The flip and flop exons encode a 38-amino-acid sequence that differs between the two isoforms by only 7 amino acids. These different isoforms show distinct cell-specific and developmental expression patterns in the brain [46, 60]. Further, several functional consequences of this alternative splicing have been identified. In general, receptors composed of flip subunits desensitize more slowly than receptors composed of flop subunits [61, 62]. AMPA receptors composed of flip or flop subunits also show differential sensitivity to positive allosteric modulation [63]. As described in further detail below, the sensitivity of AMPA receptors to positive allosteric modulation provides a mechanism by which glutamatergic synaptic transmission can be enhanced in the central nervous system and may represent a novel approach for the treatment of disease.

On the basis of recent structure–function studies, a model of the mechanisms by which AMPA receptors gate synaptic current has emerged. Evidence indicates that the tetrameric receptor is formed by dimerization of two LBCs of adjacent subunits that in turn dimerize with the dimer of the other two LBCs [64]. Activation of AMPA receptors is initiated by binding of glutamate to each of the four LBCs, leading to the closure of the upper and lower domains of each LBC which is predicted to direct a conformational change in the ion channel gate, permitting ion flux through the channel pore [64]. Termination of current flow can occur via two mechanisms. Deactivation reflects closing of the channel upon removal of glutamate and subsequent release of agonist from the LBC. Desensitization occurs in the continued presence of glutamate and reflects a destabilization and subsequent rearrangement of the intradimer interface that is predicted to uncouple the closing of the LBCs from the channel gate, permitting closure of the ion channel. Interestingly, crystallographic data have revealed that the flip/flop cassette in the S2 region is located at the intradimer interface and is associated with the “hinge” of the clamshell region of the LBC [64]. Differences in the desensitization rates of flip and flop receptors have been shown to depend on the identity of only three amino acid residues in the flip–flop region (Thr765, Pro766, and Ser775 in flip and Asn765, Ala766, and Asn775 in flop) and these three residues are postulated to confer their kinetic differences by directly and/or indirectly influencing the stability of the dimer interface between adjacent subunits [62].

The majority of neurons in the central nervous system display rapid excitatory synaptic responses to glutamatergic input indicative of AMPA receptor–dependent transmission. This observation suggests that AMPA receptors are expressed ubiquitously and uniformly throughout the central nervous system. However, although widespread, quantitative autoradiography experiments utilizing [^3H]AMPA have demonstrated a heterogeneous distribution pattern. More specifically, high levels of AMPA receptors are expressed in the hippocampal formation and the outer layers of the cerebral cortex and the dorsal lateral septum. Intermediate concentrations are observed in the striatum and deep layers of the cerebral cortex. Lower levels of expression are found in thalamic nuclei and most midbrain and brain stem regions [65]. Consistent with these findings, *in vitro* quantitative autoradiography studies using radiolabeled versions of selective AMPA receptor agonists (e.g., (*S*)-[^3H]-5-fluorowillardiine, or [^3H]FW) and competitive antagonists (e.g., 6-[^3H]nitro-7-sulphamoylbenzo[*f*]-quinoxaline-2,3-dione, or [^3H]NBQX) have revealed binding distributions similar to that of [^3H]AMPA [66–70].

10.1.5 Kainate Receptors

Kainate receptors are comprised of members of two subunit families, $\text{GLU}_{\text{K}5-7}$ and $\text{GLU}_{\text{K}1-2}$ (GluR5–7 and KA1 and KA2) [43]. Although the $\text{GLU}_{\text{K}1}$ and $\text{GLU}_{\text{K}2}$ subunits bind kainate with high affinity, they do not appear to form functional ion channels when expressed as homomers in host cells. However, they do appear to form heteromeric receptors with the $\text{GLU}_{\text{K}5-7}$ subunits. $\text{GLU}_{\text{K}5-7}$ can form homomeric receptors in recombinant systems although it is likely that native receptors are heteromers. Splice variants exist for $\text{GLU}_{\text{K}5-7}$, adding another layer of potential heterogeneity for native kainate receptors. For individual subunits, KA receptors appear to share a topographical structure similar to that described for NMDA and AMPA receptors (Fig. 10.2).

Kainate receptors are broadly distributed throughout the mammalian nervous system (reviewed by Huettner [71]). They are localized on neuronal dendrites and postsynaptic membranes as well as on nerve fibers and synaptic terminals. In general, kainate receptors play an excitatory role, existing on the postsynaptic membranes of excitatory neurons. Presynaptic kainate receptors may also modulate the release of γ -aminobutyric acid (GABA) from inhibitory neurons [71, 72].

Kainate receptors are found within both the peripheral and central nervous systems. One of the earliest observations of kainate receptor-mediated responses was the depolarization of dorsal root fibers by kainate [1, 73]. Subsequent studies have indicated the presence of kainate receptors in both dorsal root ganglion neurons and spinal cord, in particular $\text{GLU}_{\text{K}5}$ [74]. Functional kainate receptors have also been demonstrated within spinal neurons [75–77].

Other areas of prominent kainate receptor expression within the central nervous system include the basal ganglia [78], basolateral amygdala [79], sensory cortex [80], striatum [81], hippocampus (both pre- and postsynaptically) [82, 83], hypothalamus ($\text{GLU}_{\text{K}5}$ and $\text{GLU}_{\text{K}6}$) [84], and the Purkinje cells [85] and granule cell layer ($\text{GLU}_{\text{K}2}$, $\text{GLU}_{\text{K}5}$, and $\text{GLU}_{\text{K}6}$) [86, 87] of the cerebellum. Kainate receptors have also been identified in retina [88].

Kainate receptors have been implicated to play a role in certain forms of epilepsy. Kainate administration causes a well-characterized seizure syndrome in rodents, which is associated with hippocampal neurodegeneration [89, 90]. $\text{GLU}_{\text{K}6}$ knock-out mice have been shown to be resistant to kainate-evoked seizures [91]. $\text{GLU}_{\text{K}5}$ kainate receptors have also been suggested to play a role in seizures. In rats, $\text{GLU}_{\text{K}5}$ selective antagonists have been shown to prevent pilocarpine-induced limbic seizures [92].

Kainate receptors may be in pathways responsible for anxiety. Although at the present time this area of kainate receptor function has yet to be extensively explored, the mixed AMPA–kainate receptor antagonist 3*S*,4*aR*,6*R*,8*aR*-6-(2-(1(2)*H*-tetrazol-5-yl)ethyl)-decahydroisoquinoline-3-carboxylic acid (LY293558) has been shown to produce anxiolytic-like effects in some animal models [93, 94]. Based on the binding profile of LY293558 [95, 96] and other pharmacological data [94, 97], $\text{GLU}_{\text{K}5}$ -containing kainate receptors have been hypothesized to be responsible for these anxiolytic-like behavioral effects [94]. The putative role of kainate receptors in anxiety could be related to the ability of kainate receptor activation to produce an enduring enhancement of excitatory synaptic responses in the basolateral amygdala [79].

A body of evidence has been accumulated indicating that kainate receptors containing $\text{GLU}_{\text{K}5}$ subunits also play a role in pain signaling. Kainate receptor antagonists selective for $\text{GLU}_{\text{K}5}$ have been shown to be active in animal models of inflammatory hyperalgesia, peripheral neuropathy, persistent pain, and migraine [98–101]. The mixed AMPA–kainate receptor antagonist LY293558 has also been found to reduce capsaicin-evoked [102] or postoperative [103] pain as well as migraine pain [104] in humans. It is interesting to note that, while LY293558 reduces the pain associated with these pathophysiological conditions, it does not affect normal pain sensation [102]. The presence of $\text{GLU}_{\text{K}5}$ kainate receptor subunits on trigeminal neurons [105] may explain the efficacy of $\text{GLU}_{\text{K}5}$ antagonists in migraine. It is currently unknown whether the other analgesic effects of $\text{GLU}_{\text{K}5}$ antagonists are mediated by central and/or peripherally located kainate receptors.

10.2 ORTHOSTERIC PHARMACOLOGICAL AGENTS

10.2.1 NMDA Receptor Orthosteric Agonists

Although less potent in most biological studies because of its rapid uptake by transporter systems, the natural ligand L-glutamate (Fig. 10.3) has about 10-fold greater affinity for the receptor than NMDA itself. L-Aspartate (Fig. 10.3), the other potentially natural ligand for the NMDA receptor, is less selective than NMDA and is also a substrate for transport systems. Quinolinic acid, another endogenous ligand, is weaker still. Thus, because of its superior selectivity and its lack of affinity for the transporter systems, NMDA has maintained its position as the prototypical agonist for this receptor, despite both an affinity in the 10 μ M range and not being quite a full agonist. Of the older agonists, only ibotenic acid (Fig. 10.3) mimicked the sensitivity of NMDA to a panel of antagonists [2], but ibotenate displayed an inhibitory second phase, possibly due to its conversion to muscimol by endogenous enzymes [106]. Ibotenic acid has an isoxazole bioisostere for the terminal carboxyl of L-glutamate and retains a similar affinity to the natural ligand. Inorganic terminal amino acid replacements (e.g., phosphonate, phosphinate, sulfonate), however, showed no improvement over the carboxyl of L-glutamate. Interestingly, replacing this with a tetrazole (tetrazolylglycine; Fig. 10.3) led to an approximately 10-fold increase in potency with maintained selectivity for the NMDA receptor [107, 108].

Further increases in potency were obtained by conformational restriction of the open chain of glutamate. Thus the 2*S*,1'*R*,2'*S* enantiomer of cyclopropylglutamate (CCG; Fig. 10.3) has an affinity in the 10-nM range [109] and *trans*-aminocyclobutanedicarboxylate (ACBD; Fig. 10.3) in the 100-nM range [110] similar to that of tetrazolylglycine [108]. Despite improved potency, these agonists have not become popular replacements for NMDA in pharmacological studies, presumably because of the more difficult synthesis and hence reduced availability.

The above glutamate site agonists act promiscuously at all the GLU_{N2} subtypes and no appreciable subtype selectivity has been reported. Homoquinolinic acid (Fig. 10.3), in which the amino group is incorporated into a pyridine ring, does, however, show such preferential selectivity for GLU_{N2B} subunits [47, 48], although the selectivity may not be so great at human GLU_{N2} subtypes [111].

10.2.2 NMDA Receptor Orthosteric Antagonists

Pharmacologically useful NMDA antagonists were initially developed by replacing the terminal carboxylate of D- α -amino adipate with a phosphonate to make D-2-amino-5-phosphonopentanoate (D-AP5; originally known as D-APV) [24] and by extending the chain length by two carbons to make D-2-amino-7-phosphonoheptanoate (Fig. 10.4; D-AP7). Interestingly compounds with intermediate lengths of this carbon backbone, as in D-AP4, D-AP6, and D-AP8, were relatively inactive as NMDA antagonists [24]. Compounds with the terminal amino acid of D-AP5 and D-AP7 replaced with a tetrazole and a carboxyl moiety still retain antagonist activity but the α -carboxyl cannot be replaced by phosphonate. The discovery of D-AP5 and D-AP7 catalyzed a large number of experiments to investigate the physiological and pathological functions of the NMDA receptor. For example, D-AP5 was used to determine the role of NMDA receptors in synaptic transmission and in long-term

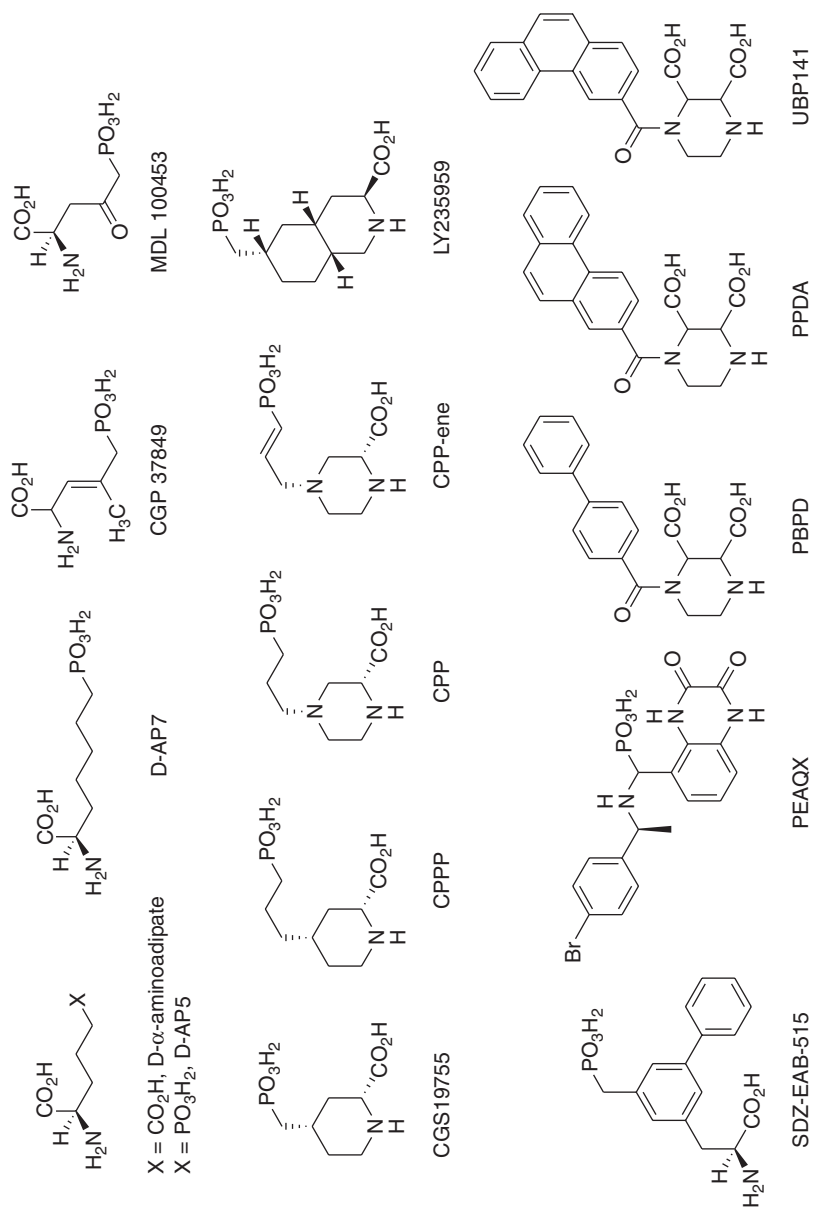


Figure 10.4 NMDA receptor orthosteric antagonists.

potentiation (LTP) [7, 25], later shown to translate into memory defects in vivo [112]. D-AP5 and D-AP7 were shown to reduce neuronal cell death in animal models of cerebral ischaemia [26, 113] and reduce convulsions initiated in a number of different ways [26, 27] (see also [30]).

These compounds, however, only accessed the central nervous system poorly because of their polar nature—a severe limitation on their usefulness in behavioral and other in vivo studies and for therapeutic utility. In an attempt to improve blood–brain barrier penetration, by decreasing polarity and improving potency, many structurally constrained molecules were made, particularly by pharmaceutical companies (see [114, 115]). Thus, about an order-of-magnitude increase in potency over D-AP5 was achieved by introducing a double bond (CGP 37849; Fig. 10.4) [116] or a keto group (MDL 100453; Fig. 10.4 [117] or heteroatoms [118] into the carbon backbone. However, to achieve brain penetration following oral administration, esterification of the α -carboxyl of CGP 37849 was needed [116]. Further constraint of the carbon chain was gained by including the α -carboxyl of D-AP5 and D-AP7 in a piperidine (CGS19755 and CPPP; Fig. 10.3) [119] or piperazine (CPP; Fig. 10.4) [120] ring structure. Again the inclusion of a double bond or keto group improved potency as in CPP-ene (Fig. 10.4) [121] or MDL 100925 [122], respectively. The ultimate in structural constraint was achieved by incorporating a second ring into CPP. The resultant compound, LY235959 (Fig. 10.4), is among the most potent competitive NMDA receptor antagonists reported with an affinity in the 10nM range, approximately 100-fold more potent than the parent, AP7 [123, 124]. Biphenyl side chains have also been substituted in the D-AP7 molecule, for example SDZ-EAB-515 (Fig. 10.4), resulting in potent compounds with increased bioavailability and reasonable potency.

Several of the above compounds have been in development as potential therapies for epilepsy, [e.g., CPPene (SDZ-EAA-494)] and for stroke [e.g., CGS19755 (Selfotel)], but none are still in clinical development [29]. There have been a number of issues relating to dose levels, timing of the first treatment for stroke, and particularly appearance of adverse central nervous system side effects which, coupled with lack of efficacy in the outcomes measured, have led to the withdrawal of these compounds (see [28, 125]). It is generally assumed that these compounds show little if any selectivity between $\text{GLU}_{\text{N}2}$ subtypes. It has been reported, however, that D-CPPene shows preference for $\text{GLU}_{\text{N}2\text{A}}$ and $\text{GLU}_{\text{N}2\text{B}}$ over $\text{GLU}_{\text{N}2\text{C}}$ and $\text{GLU}_{\text{N}2\text{D}}$, whereas SDZ-EAB-515 and PBPD (Fig. 10.4) are less active on the $\text{GLU}_{\text{N}2\text{A}}$ subtype [48]. This selectivity correlates with the regional variation in antagonist affinity and the known distribution of $\text{GLU}_{\text{N}2}$ subtypes [48]. The competitive antagonist CPP displays a 50-fold selectivity for $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{D}}$ but only 6- to 7-fold selectivity for $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{B}}$ [126]. The longer chain antagonists such as CPP have been shown to display greater selectivity for $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{C}}$ or $\text{GLU}_{\text{N}2\text{D}}$ than the shorter chain antagonists such as D-AP5 or CGS19755 [126]. A new compound, PEAQX, or AAM077 (Fig. 10.4), is claimed to show a 100-fold selectivity for human $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{B}}$ [127]. However, when tested on rat subunits the selectivity was shown to be only 10-fold for $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{B}}$ and 2- to 3-fold for $\text{GLU}_{\text{N}2\text{A}}$ over $\text{GLU}_{\text{N}2\text{C}}$ or $\text{GLU}_{\text{N}2\text{D}}$ [49]. Additionally, a recent report, suggests that the order of antagonist/agonist application affects the apparent subunit selectivity of this compound [128]. Recently, two compounds, PPDA and UBP141 (Fig. 10.4), have been reported to show 3- to 7-fold selectivity for $\text{GLU}_{\text{N}2\text{D}}$ over $\text{GLU}_{\text{N}2\text{A}}$ [49, 129].

These compounds have the reverse selectivity ($\text{GLU}_{\text{N2C}}/\text{GLU}_{\text{N2D}}$ over $\text{GLU}_{\text{N2A}}/\text{GLU}_{\text{N2B}}$) compared to the majority of competitive NMDA receptor antagonists, which show $\text{GLU}_{\text{N2A}}/\text{GLU}_{\text{N2B}}$ selectivity.

In contrast to their effects at the polyamine site, conantokin-G peptide, antagonism of NMDA receptors has also been suggested to be via the glutamate recognition site, with a strong preference for GLU_{N2B} subunit containing receptors [130].

10.2.3 AMPA Receptor Orthosteric Agonists

AMPA and analogs: A large number of orthosteric agonists of AMPA receptors have been described and many of them have been derived from classic structure–activity relationship studies using AMPA as a lead molecule (Fig. 10.5) [131]. A variety of analogs of AMPA having different substituents in the 5 position of the isoxazole ring have been synthesized which exhibit potent agonist properties, including the trifluoro AMPA and the phenyl derivative (*S*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl) propionic acid [(*S*)APPA] (for review see [132]). Additional AMPA analogs include the phenyl carboxy derivative (*R,S*)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl) propionic acid [ACPA] [133] and 4-AHCP [134].

Willardiines and natural products: Another structurally distinct class of AMPA receptor agonists originates from willardiine. Among the willardiine agonists, a series of 5-substituted (*S*)-willardiines have been evaluated at native AMPA receptors on hippocampal neurons and display a rank order potency of fluoro > cyano = trifluoromethyl = nitro > chloro = bromo > (*R,S*)-AMPA > iodo > willardiine [135]. In addition to their activity at AMPA receptors, some willardiine derivatives (e.g., 5-iodowillardiine) also display potent activity at kainate receptors [135].

Naturally occurring neurotoxins such as domoic acid, a rigid analog of glutamate originally isolated from the macroscopic red alga *Chondria armata* (known in Japan as domoi), also have been shown to be potent agonists of AMPA receptors [136]. As well, kainic acid isolated from the dinoflagellate *Digenea simplex*, in addition to its agonist activity at kainate receptors, is an agonist of AMPA receptors [137, 138]. Both of these compounds are also agonists of kainate receptors and are discussed in greater detail below.

AMPA receptor agonists display a wide range of peak, steady-state whole-cell currents that are also reflected in corresponding single-channel currents. For example, saturating concentrations of glutamate and AMPA evoke large inward currents that desensitize rapidly to levels $\leq 10\%$ of the peak current depending on the receptor subtype [62, 63, 139, 140]. In contrast, kainate and domoate produce smaller

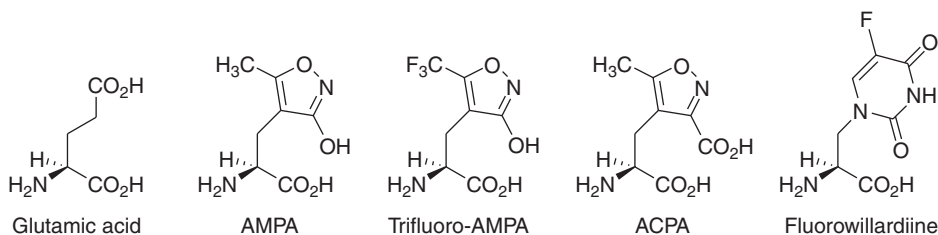


Figure 10.5 AMPA receptor orthosteric agonists.

peak currents and desensitize to levels substantially less ($\sim 70\%$ of peak current) than AMPA or glutamate [137, 138, 141]. Similarly, single-channel analyses have shown that kainate elicits significantly smaller currents than glutamate or AMPA, confirming its partial agonist nature at AMPA receptors [142]. The structural basis for differences between full and partial agonists has been elucidated in crystallographic studies analyzing the LBC of GLU_{A2} in the presence of glutamate, AMPA, and kainate [55]. The extent of agonism was shown to be directly related to the degree of closure of the upper and lower domains of the LBC such that full agonists induce greater domain closure than partial agonists. Functionally, the intermediate domain closure of kainate likely leads to a partial conformational change at the channel gate. Differences in the degree of desensitization between full and partial agonists are predicted to arise from differences in the extent of domain closure which affects the degree of intersubunit dimerization [55].

10.2.4 AMPA Receptor Orthosteric Antagonists

Quinoxalines: The first potent competitive antagonists of AMPA receptors were quinoxaline analogs, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitro-quinoxaline-2,3-dione (DNQX) (Fig. 10.6) [143, 144]. Although these compounds were not selective, having antagonist activity at the glycine binding site on NMDA receptors [145], they were prototypes for additional quinoxaline antagonists lacking the glycine site interaction, such as 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) [146]; 1,4,7,8,9,10-hexahydro-9-methyl-6-nitropyrido[3,4-*f*]-quinoxaline-2,3-dione (PNQX); 6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*H*,4*H*)-quinoxalinedione (YM-90K; Fig. 10.6) [147]; [2,3-dioxo-7-(1*H*-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxaliny]-acetic acid (YM872) [148]; and [1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methylphosphonate (ZK200775) [149]. However, these newer compounds (as well as the older quinoxalines) also antagonize kainate receptors with varying degrees of selectivity for AMPA receptors, ranging from 3- to 10-fold [150].

Decahydroisoquinolines: Recently, other competitive antagonists belonging to the class of decahydroisoquinoline compounds have been discovered [151]. For example, LY293558 was shown to selectively displace ^3H -AMPA and ^3H -CNQX binding to rat brain membranes and potently antagonize AMPA-evoked depolarizations in rat cortical slices in a competitive manner (see Fig. 10.8 below) [95]. However, similar to the quinoxalines, the degree of selectivity for AMPA receptors versus kainate receptors of decahydroisoquinolines can vary considerably [96, 151–153]. For instance, LY293558 binds with equal affinity to AMPA ($K_D = 3.3 \mu\text{M}$) and kainate ($K_D = 4.9 \mu\text{M}$) receptors, whereas LY382884 binds with at least 100-fold greater

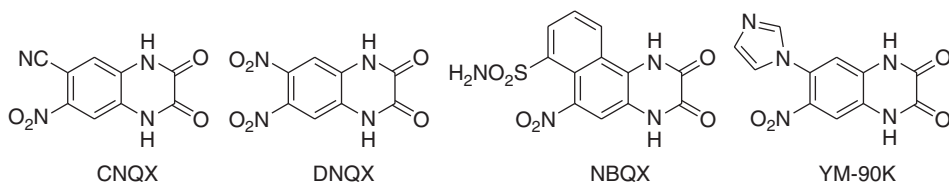


Figure 10.6 AMPA receptor orthosteric antagonists.

affinity to kainate ($K_D = 3.6 \mu\text{M}$) versus AMPA ($K_D = 550 \mu\text{M}$) receptors [153]. Other decahydroisoquinolines show slight selectivity for AMPA receptors. For instance, LY302679 is approximately fourfold selective for AMPA ($K_i = 0.8 \mu\text{M}$) versus kainate ($K_i = 3.4 \mu\text{M}$) receptors [153].

An understanding of the mechanism of orthosteric antagonism of AMPA receptors has come from crystal structures of the AMPA receptor LBC in the presence of DNQX [55]. These studies showed that competitive antagonists act by preventing closure of the two domains of the LBC. Indeed, although not identical, the degree of domain closure of the LBC in the DNQX crystals is quite similar to that observed in the agonist unbound apo state crystals. Thus, in the presence of a competitive antagonist, stabilization of the “open” configuration of the two domains of the LBC is predicted to preclude a directed conformational change at the channel gate [55].

10.2.5 Kainate Receptor Orthosteric Agonists

Glutamate: Glutamate, the endogenous ligand, activates kainate receptor channels with median effective concentration (EC_{50}) values at recombinant human kainate receptors ranging from approximately $10 \mu\text{M}$ for GLU_{K6} and $\text{GLU}_{K2}/\text{GLU}_{K6}$, to $\sim 50 \mu\text{M}$ for GLU_{K5} and $\text{GLU}_{K5}/\text{GLU}_{K6}$ [97]. Glutamate exhibits much lower affinity for GLU_{K7} ($EC_{50} = 6 \text{ mM}$ [154]). Obviously, glutamate is not selective for kainate receptors as it also activates all other ionotropic and metabotropic glutamate receptors.

Gamma glutamate analogs: The gamma-substituted glutamate analog (2*S*,4*R*)-4-methylglutamate (SYM2081) has been studied at GLU_{K5} - and GLU_{K6} -containing channels. SYM2081 activates these channels with sub-micromolar (0.06 – $0.4 \mu\text{M}$) potency (Fig. 10.7) [97]. SYM2081 produces pronounced desensitization and acts as a functional antagonist with prolonged application. Presumably because of its ability

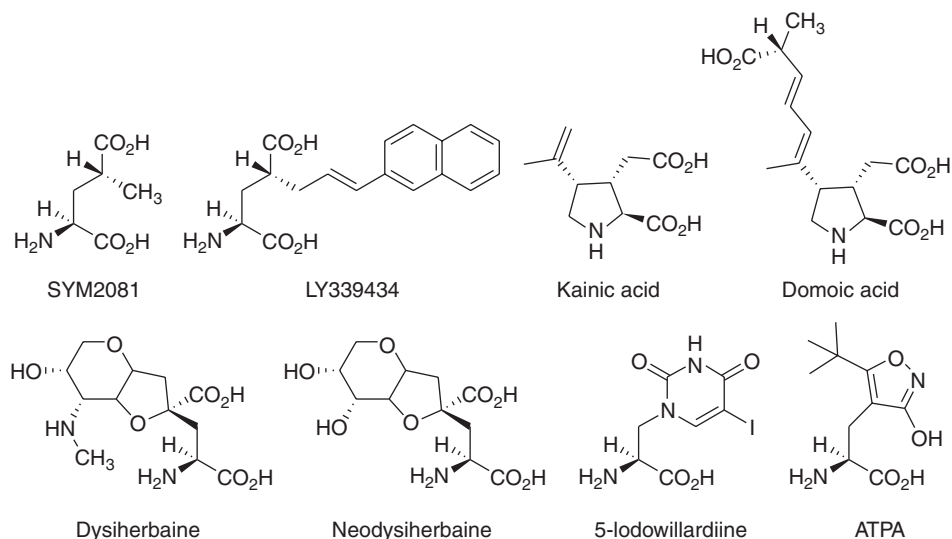


Figure 10.7 KA receptor orthosteric agonists.

TABLE 10.1 Agonist Potency at Recombinant Human GLU_{K5}- and GLU_{K6}-Containing Kainate Receptors

Agonist	GLU _{K5}	GLU _{K6}	GLU _{K5} /GLU _{K6}	GLU _{K2} /GLU _{K5}	GLU _{K2} /GLU _{K6}
Glutamate	47	9	48	19	8
Kainate	5	1	7	2	0.6
SYM2081	0.2	0.3	0.4	0.06	0.3
Domoate	0.4	0.07	0.2	0.05	0.1
AMPA	208 ^a	NA (1 mM)	154 ^a	123 ^a	137 ^a
ATPA	0.3	NA (0.1 mM)	0.8	0.4	106 ^a
Iodowillardiine	0.2	NA (1 mM)	0.5	0.06	30 ^a

Note: Values given represent EC₅₀ (in μ M) determined in the presence of concanavalin A.

Source: From [97].

^aPartial agonist. NA = no agonist activity observed (up to the concentration listed).

to functionally antagonize GLU_{K5} channels, SYM2081 produces antagonist-like antinociceptive responses in animal models of nerve injury [155]. SYM2081 is selective for kainate versus AMPA receptors but does not display pharmacological selectivity between GLU_{K5} and GLU_{K6} receptors (Table 10.1) [97, 156]. SYM2081 also exhibits agonist activity at NMDA receptors (EC₅₀ = 12 μ M at NMDA receptors expressed natively in cultured rat hippocampal neurons [156]).

Related analogs such as (2*S*,4*R*,6*E*)-2-amino-4-carboxy-7-(2-naphthyl)hept-6-enoic acid (LY339434) show improved selectivity for GLU_{K5} versus GLU_{K6} receptors (Fig. 10.7) [156]. LY339434 was found to evoke inward currents at recombinant human GLU_{K5} with EC₅₀ = 2.5 μ M but had little effect at GLU_{K6} at concentrations less than 100 μ M [156]. Like SYM2081, LY339434 shows low potency at AMPA receptors (EC₅₀ > 300 μ M) but is a potent agonist at NMDA receptors (EC₅₀ = 2.5 μ M at NMDA receptors expressed natively in cultured rat hippocampal neurons [156]). Related alkylidene glutamate analogs also are potent GLU_{K5} agonists with increased binding affinity produced by the introduction of lipophilic 4-alkylidene groups. In contrast, potencies of these agonists at GLU_{K6} receptors were decreased by this functional group [157].

The selectivity of the γ -glutamate analogs for GLU_{K5} versus GLU_{K6} receptors has been used to provide evidence that the kainate receptors expressed in cultured hippocampal rat neurons represent primarily GLU_{K6} receptors [158].

Marine products: Kainate is derived from the seaweed *D. simplex*. Domoate, another kainate receptor agonist, originates from the related seaweed *C. armate* (Fig. 10.7). Both compounds are potent neurotoxins. Kainate activates GLU_{K5} and GLU_{K6} receptors with EC₅₀ values in the low (\sim 1–10) micromolar range. Domoate is more potent, with EC₅₀ values ranging from \sim 0.05 to 0.4 μ M [97]. Like glutamate, kainate is able to activate GLU_{K7} receptors, but with extremely low (millimolar range) potency, while domoate is not active at GLU_{K7} [154]. Both of these agonists also evoke non-desensitizing responses at AMPA receptors, limiting their use as kainate-selective ligands. However, low concentrations of domoate (0.2 μ M) have been used to selectively activate kainate receptors [159]. Interestingly, domoate-evoked activation of kainate receptor channels appears to be less subject to desensitization than that produced by other kainate receptor agonists. Therefore, domoate has been used in the

absence of concanavalin A in assays which typically require the use of the lectin (or another blocker of desensitization) in order to observe agonist effects [160].

Dysiherbaine, another potent neuroexcitotoxin, was isolated from the Micronesian sponge *Dysidea herbacea* (Fig. 10.7) [161]. In rat brain synaptic membranes, dysiherbaine was found to displace [^3H]kainate and [^3H]AMPA binding with K_i values of 26 and 153 nM, respectively [162]. Dysiherbaine did not displace the binding of the NMDA receptor ligand [^3H]CGS-19755. Dysiherbaine displaced [^3H]kainate at recombinant rat kainate receptors with higher affinity than was observed in native tissues, with K_i values of 4, 0.5, and 1 nM at recombinant rat $\text{GLU}_{\text{K}2}$, $\text{GLU}_{\text{K}5}$, $\text{GLU}_{\text{K}6}$, respectively [163]. In whole-cell voltage clamp recordings from cultured rat hippocampal neurons, dysiherbaine evoked inward currents from both kainate and AMPA receptors with EC_{50} values of 0.2 and 9.7 μM , respectively. In calcium imaging experiments, it was found that dysiherbaine also activated the group I metabotropic receptor mGluR5 [162]. Dysiherbaine appears to bind to $\text{GLU}_{\text{K}2}$ subunits with lower affinity than $\text{GLU}_{\text{K}5}$ or $\text{GLU}_{\text{K}6}$ subunits and has been used to provide evidence that kainate receptor current may be gated by individual subunits [164].

Neodysiherbaine (Fig. 10.7) is a natural analog of dysiherbaine that is produced by *D. herbacea* in much lower quantities [165]. Like dysiherbaine, neodysiherbaine is an agonist at both kainate and AMPA receptors [163]. In radioligand binding assays using recombinant rat receptors, neodysiherbaine displaced [^3H]kainate with 7-fold higher affinity than dysiherbaine at $\text{GLU}_{\text{K}2}$ receptors but displayed 15- to 25-fold lower affinity than dysiherbaine at $\text{GLU}_{\text{K}5}$ and $\text{GLU}_{\text{K}6}$ receptors (K_i values of 0.6, 7.7, and 33 nM for neodysiherbaine at $\text{GLU}_{\text{K}2}$, $\text{GLU}_{\text{K}5}$, and $\text{GLU}_{\text{K}6}$ receptors, respectively [163]). Neodysiherbaine shows no affinity for NMDA receptors [165], and, unlike dysiherbaine, neodysiherbaine shows no agonist activity at mGluR5 receptors [163].

The synthetic dysiherbaine analog (2*R*,3*aR*,7*aR*)-2-[(2*S*)-2-amino-2-carboxyethyl]-hexahydro-furo[3,2-*b*]pyran-2-carboxylic acid (MSVIII-19) can act as a functional antagonist (a desensitizing agonist) at $\text{GLU}_{\text{K}5}$ receptors and, with much lower affinity, AMPA receptors [163]. MSVIII-19 displaced [^3H]kainate from recombinant rat $\text{GLU}_{\text{K}5}$ with $K_i = 0.1 \mu\text{M}$. MSVIII-19 displays no appreciable affinity for $\text{GLU}_{\text{K}2}$ or $\text{GLU}_{\text{K}6}$ receptors ($K_i > 100 \mu\text{M}$) [163].

Willardiines: (S)-1-(2-Amino-2-carboxyethyl)pyrimidine-2,4-dione, or (S)-willardiine, occurs naturally in the seeds of several species of shrub trees [166]. Willardiine and a series of 5-substituted (S)-willardiine analogs were discovered to act as agonists at AMPA and kainate receptors [167]. Their rank order of potency in rat dorsal root ganglia (DRG) neurons, a $\text{GLU}_{\text{K}5}$ -containing preparation, is trifluoromethyl ($\text{EC}_{50} = 70 \text{ nM}$) > iodo > bromo \approx chloro > nitro \approx cyano > methyl > fluoro \gg willardiine ($\text{EC}_{50} = 69 \mu\text{M}$) [135]. In addition to differences in their potency and selectivity for $\text{GLU}_{\text{K}5}$ versus AMPA receptors, these willardiine analogs also vary dramatically in their desensitization kinetics ($\tau_{\text{off}} = 43 \text{ ms}$ for 5-fluoro versus 4.2 s for 5-iodo).

Of the willardiine analogs, (S)-5-iodowillardiine has been the most extensively studied. (S)-5-Iodowillardiine displays 100- to 1000-fold selectivity for $\text{GLU}_{\text{K}5}$ versus AMPA receptors (Fig. 10.7) [135, 168] and shows no appreciable activity at recombinant human homomeric $\text{GLU}_{\text{K}6}$ or $\text{GLU}_{\text{K}7}$ receptors [97, 169]. Interestingly, (S)-5-iodowillardiine has been shown to function as a partial agonist at heteromeric $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ [97, 169] or $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}7}$ [142] receptors. More recently, a

number of willardiine derivatives which act as competitive $\text{GLU}_{\text{K}5}$ antagonists have also been identified (discussed below).

AMPA and ATPA: In addition to potently activating AMPA receptors, AMPA also activates $\text{GLU}_{\text{K}5}$ -containing channels. Alt et al. [97] reported that AMPA functions as a partial agonist at recombinant human homomeric $\text{GLU}_{\text{K}5}$ and heteromeric $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ and $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$ kainate receptors with EC_{50} values of 0.1–0.2 mM. Like (*S*)-5-iodowillardiine, AMPA has no effect at homomeric $\text{GLU}_{\text{K}6}$ receptors but displays partial agonist activity at recombinant human $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ receptors ($\text{EC}_{50} = 30 \mu\text{M}$ [97]).

The *tert*-butyl analog of AMPA, (*R,S*)-2- α -amino-3-hydroxy-5-*tert*-butyl-4-isoxazolepropionic acid (ATPA), was originally developed as an AMPA receptor agonist. ATPA has greater brain penetration than AMPA but has lower affinity for AMPA receptors (Fig. 10.7) [170]. Using cell lines stably expressing recombinant human glutamate receptors, Clarke et al. [152] found that ATPA binds selectively to $\text{GLU}_{\text{K}5}$ ($K_i = 4.3 \text{ nM}$) versus $\text{GLU}_{\text{A}1-4}$ (K_i values of 6–14 μM), $\text{GLU}_{\text{K}2}$ or $\text{GLU}_{\text{K}7}$ ($K_i > 10 \mu\text{M}$), or $\text{GLU}_{\text{K}6}$ or heteromeric $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ ($K_i > 1 \text{ mM}$) receptors. In whole-cell recordings, ATPA evoked inward currents at $\text{GLU}_{\text{K}5}$ ($\text{EC}_{50} = 2 \mu\text{M}$) and AMPA (EC_{50} values of 0.4–0.7 mM) receptors but showed no agonist activity at $\text{GLU}_{\text{K}6}$ receptors at concentrations up to 10 mM [152]. Alt et al. [97] reported similar EC_{50} values for ATPA for evoking calcium influx through recombinant human $\text{GLU}_{\text{K}5}$, $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$, or $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ receptors ($\text{EC}_{50} = 0.3\text{--}0.8 \mu\text{M}$) or for evoking inward currents at recombinant human $\text{GLU}_{\text{K}5}$ or $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ ($\text{EC}_{50} = 1 \mu\text{M}$) or $\text{GLU}_{\text{K}5}$ receptors natively expressed in rat DRG ($\text{EC}_{50} = 0.4 \mu\text{M}$). Additionally, in a calcium influx assay, ATPA was found to function as a partial agonist at heteromeric $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ receptors ($\text{EC}_{50} = 0.1 \text{ mM}$), and it was demonstrated that ATPA partially antagonized the agonist effects of glutamate at these channels. Similarly, at homomeric $\text{GLU}_{\text{K}6}$ receptors, where ATPA showed no agonist activity, it was found that ATPA was able to block calcium influx evoked by glutamate, which would seemingly indicate that ATPA does in fact bind to $\text{GLU}_{\text{K}6}$ receptors, albeit with low affinity (median inhibitory concentration $\text{IC}_{50} \sim 2 \text{ mM}$, corresponding to a K_b of approximately 0.2 mM [97]).

10.2.6 Kainate Receptor Orthosteric Antagonists

Oxime derivatives: One of the first compounds identified as a kainate receptor antagonist was the oxime derivative 5-nitro-6,7,8,9-tetrahydrobenzo[g]indole-2,3-dione-3-oxime (NS102; Fig. 10.8). It is a weak antagonist at $\text{GLU}_{\text{K}6}$ receptors [171]. It also antagonized $\text{GLU}_{\text{K}5}$ receptors expressed in rat DRG neurons ($K_b = 6 \mu\text{M}$ [172]). NS102 is also an AMPA receptor antagonist, blocking AMPA receptor-mediated responses in primary rat cortical cultures with $K_b = 114 \mu\text{M}$ [172].

Recently, another oxime derivative, 8-methyl-5-(4-(*N,N*-dimethylsulfamoyl)phenyl)-6,7,8,9-tetrahydro-1*H*-pyrrolo[3,2-*h*]-iso-quinoline-2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime (NS1209), has been shown to be effective in the mouse hot-plate test (an acute-pain model), the rat formalin test (a model of persistent pain), and the rat chronic constriction injury model of neuropathic pain [173]. However, NS1209 exhibits antagonist activity at both AMPA and $\text{GLU}_{\text{K}5}$ receptors (with approximately 30-fold selectivity for AMPA receptors [174]), making it difficult to conclude which receptor type mediated these pharmacological effects.

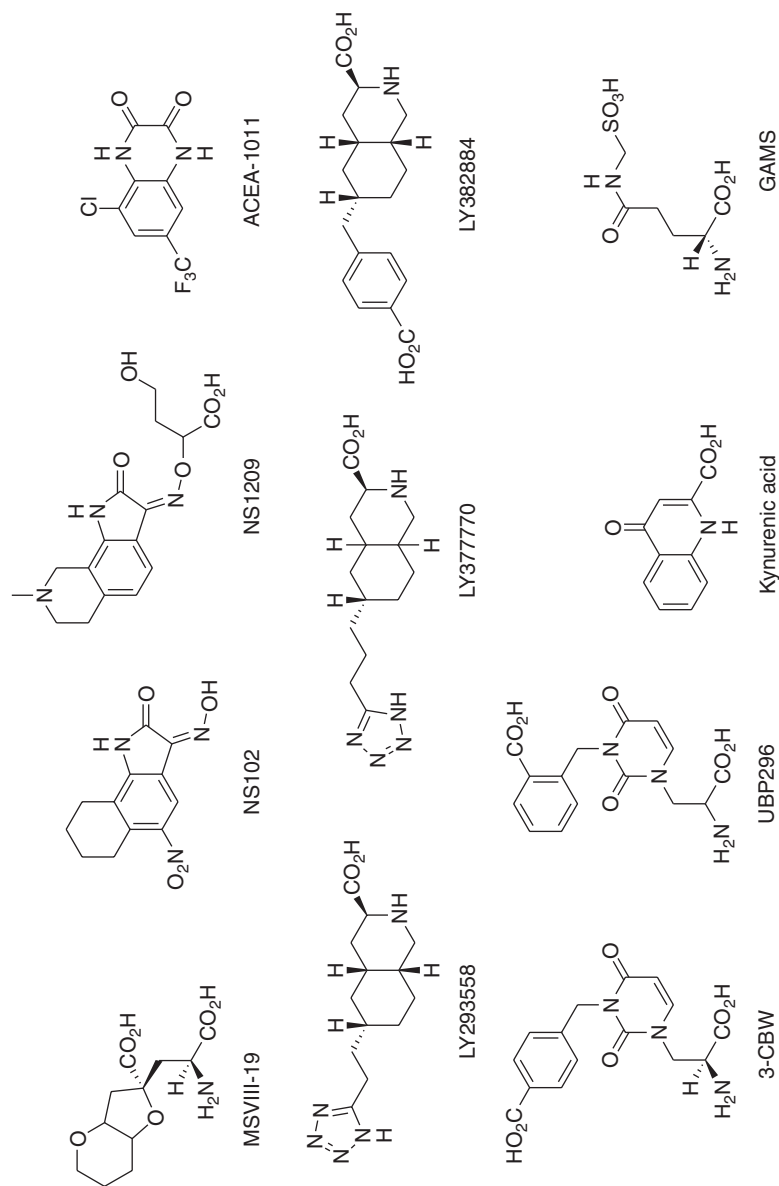


Figure 10.8 KA receptor orthosteric antagonists.

Quinoxalinediones: A number of quinoxaline derivatives have been discovered to function as both AMPA and kainate receptor antagonists. Wilding and Huettner [175] examined a series of these compounds and compared their ability to block inward currents evoked by kainate (which also activates AMPA receptors) in primary cultures of either rat DRG (a GLU_{K5} -containing preparation) or cortex (an AMPA receptor-containing preparation). Of the compounds tested, NBQX showed the greatest (3-fold) selectivity for AMPA receptors, while 5-chloro-7-trifluoromethyl-2,3-quinoxalinedione (ACEA-1011) displayed the greatest (12-fold) selectivity for kainate versus AMPA receptors. However, this compound exhibits even greater potency ($K_b = \sim 0.5 \mu\text{M}$ [176, 177]) at NMDA receptors than that observed for kainate receptors ($K_b = 1 \mu\text{M}$ [175]).

Of the quinoxalinediones, NBQX and CNQX are the most extensively studied, primarily as AMPA receptor antagonists, although both are also kainate receptor antagonists and CNQX is an antagonist at NMDA receptors. NBQX and CNQX block AMPA receptor-mediated responses in cortical preparation with K_b values of 0.3 and $1.3 \mu\text{M}$, respectively [175]. At kainate receptors, both NBQX and CNQX display little selectivity across kainate receptor subtypes [97]. The antagonist potencies for NBQX and CNQX at recombinant human kainate receptors are given in Table 10.2.

Decahydroisoquinolines: The first decahydroisoquinolines described were NMDA-selective antagonists [178]. In the course of structure-activity studies on this series, decahydroisoquinolines which functioned as AMPA receptor antagonists were identified [151]. LY293558 was the first decahydroisoquinoline discovered to have potent GLU_{K5} antagonist activity, in addition to functioning as an AMPA/NMDA

TABLE 10.2 Antagonist Potency at Recombinant Human GLU_{K5} - and GLU_{K6} -Containing Kainate Receptors

Antagonist	GLU_{K5}	GLU_{K6}	$\text{GLU}_{\text{K5}}/\text{GLU}_{\text{K6}}$	$\text{GLU}_{\text{K2}}/\text{GLU}_{\text{K5}}$	$\text{GLU}_{\text{K2}}/\text{GLU}_{\text{K6}}$
LY293558	0.2	NA (0.1 mM)	0.6	0.8	NA (0.1 mM)
LY377770	0.06	NA (0.1 mM)	0.1	0.2	NA (0.1 mM)
LY382884	0.6	NA (0.1 mM)	1	0.6	NA (0.1 mM)
CNQX	3	1	1	2	5
NBQX	8	2	6	4	6
Kynurenic acid	133	32	156	99	NA (3 mM)
GAMS	~ 300	NA (3 mM)	~ 300	~ 300	NA (3 mM)
UBP296	0.6^a	NA (0.3 mM) ^a	0.8^a	1^a	NA (0.3 mM) ^a
NS3763 ^b	1.6^c	NA (30 μM) ^c			
AUBA ^d	1.2^e	62^e			

Note: Values given represent K_b (in μM) for inhibition of agonist-induced responses in the presence of concanavalin A and are calculated from [97] unless otherwise noted. NA = no antagonist activity observed (up to the concentration listed).

^aFrom [183].

^b5-carboxyl-2,4-di-benzamido-benzoic acid.

^c IC_{50} value (noncompetitive antagonist). Determined in the absence of concanavalin A [160].

^d2-Arylureidobenzoic acid.

^e IC_{50} value (noncompetitive antagonist). Determined in the absence of concanavalin A [183a].

antagonist [95]. Although LY293558 shows virtually no selectivity for $\text{GLU}_{\text{K}5}$ versus AMPA receptors and modest (~ 10 -fold) selectivity for $\text{GLU}_{\text{K}5}$ versus NMDA receptors [179], it displays virtually complete selectivity for $\text{GLU}_{\text{K}5}$ versus $\text{GLU}_{\text{K}6}$ [96].

Other decahydroisoquinolines were subsequently discovered which exhibited even greater potency, such as LY377770 (Table 10.2). Others showed greatly improved selectivity for kainate versus AMPA receptors while maintaining selectivity for $\text{GLU}_{\text{K}5}$ versus $\text{GLU}_{\text{K}6}$. 3*S*,4*aR*,6*S*,8*aR*-6-((4-carboxyphenyl)methyl)-1,2,3,4,4*a*,5,6,7,8,8*a*-decahydroisoquinoline-3-carboxylic acid (LY382884) was found to inhibit radioligand binding to recombinant human $\text{GLU}_{\text{K}5}$ with $K_i = 4 \mu\text{M}$, while showing little or no affinity ($K_i > 100 \mu\text{M}$) for $\text{GLU}_{\text{A}1-4}$, $\text{GLU}_{\text{K}2}$, $\text{GLU}_{\text{K}6}$, $\text{GLU}_{\text{K}7}$, or a heteromeric assembly of $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}7}$. Additionally, LY382884 had little activity at NMDA receptors, producing only a small effect at $300 \mu\text{M}$ [180]. Like other $\text{GLU}_{\text{K}5}$ -selective antagonists, LY382884 has also been found to be a potent antagonist of heteromeric $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ receptors [97, 180].

A great deal of evidence for the involvement of $\text{GLU}_{\text{K}5}$ receptors in pathophysiological pain states has come from experiments using decahydroisoquinolines. LY293558 was found to reduce capsaicin-evoked hyperalgesia in the skin of human subjects without affecting normal pain sensation [102]. LY293558 has also been found to reduce spontaneous and movement-evoked pain following oral surgery [103] and to reduce migraine pain in humans [102]. In rats, Simmons et al. [98] showed that LY382884 was efficacious in the formalin test, an animal model of persistent pain. Palacek et al. [100] found that LY382884 attenuated the responses of spinothalamic tract neurons to mechanical and thermal stimuli in a model of peripheral neuropathy using anesthetized monkeys.

Recent research on decahydroisoquinolines has focused on the creation of prodrug molecules with improved oral bioavailability. In this regard, ester prodrugs of decahydroisoquinolines have recently been shown to be orally active in animal models of migraine, inflammatory hyperalgesia, and persistent pain [99, 101].

Willardiine derivatives: As mentioned above, (*S*)-willardiine and a number of 5-substituted (*S*)-willardiine analogs act as agonists at AMPA and kainate receptors. The N^3 -substituted willardiine analog (*S*)-3-(4-carboxybenzyl)willardiine (3-CBW) was found to act as an antagonist at AMPA and kainate receptors, expressed in rat neuronal tissues [181]. 3-CBW (Fig. 10.8) antagonized kainate-induced responses on rat dorsal root C fibers (presumably $\text{GLU}_{\text{K}5}$ receptors) with $\text{pA}_2 = 4.96$ (corresponding to an apparent K_D of approximately $11 \mu\text{M}$) based on Schild plot analysis. The pA_2 value at AMPA receptors was 4.48 (corresponding to a K_D of approximately $33 \mu\text{M}$).

Structure-activity studies on this series led to the identification of compounds with greater potency and selectivity for kainate receptors versus AMPA receptors [182]. (*R,S*)-3-(2-Carboxybenzyl)willardiine (UBP296) was been identified as a member of this series which displayed very potent (approximately $1 \mu\text{M}$) affinity for $\text{GLU}_{\text{K}5}$ receptors, exhibited selectivity for kainate versus AMPA receptors, and showed no significant activity at NMDA receptors or group I metabotropic glutamate receptors (mGlu_1 or mGlu_5 [183]). UBP296 is very selective for $\text{GLU}_{\text{K}5}$ versus $\text{GLU}_{\text{K}6}$. The *S* enantiomer UBP302 was shown to have the predominant antagonist activity at native $\text{GLU}_{\text{K}5}$ kainate receptors ($K_D = 0.4 \mu\text{M}$) [183, 184]. In human embryonic kidney (HEK) 293 cells recombinantly expressing human kainate receptors, UBP296

antagonized 100 μM glutamate at homomeric $\text{GLU}_{\text{K}5}$ or heteromeric $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$ channels with K_{b} values of 0.6 and 1 μM , respectively, but had no effect at $\text{GLU}_{\text{K}6}$ or $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ receptors at concentrations up to 300 μM (Table 10.2). Like other $\text{GLU}_{\text{K}5}$ -selective antagonists, UBP296 was an antagonist at heteromeric $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ receptors with potency similar to that seen at homomeric $\text{GLU}_{\text{K}5}$ ($\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ at $K_{\text{b}}=0.8 \mu\text{M}$). UBP296 also exhibits low affinity binding to recombinant human $\text{GLU}_{\text{K}7}$, displacing [^3H]kainate with $K_{\text{i}}=0.9 \text{ mM}$ [184].

Kynurenic acid: A tryptophan metabolite, kynurenic acid is a low-affinity antagonist at AMPA, NMDA, and kainate receptors (Fig. 10.8) [185]. Kynurenic acid was found to block glutamate (100 μM)–evoked calcium influx through recombinant human kainate receptors with IC_{50} values of approximately 500 μM for $\text{GLU}_{\text{K}5}$, $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$, $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$, and $\text{GLU}_{\text{K}6}$ (Table 10.2) [97]. Interestingly, kynurenic acid had no discernable activity at the $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ receptors at concentrations up to 3 mM.

GAMS: γ -D-Glutamylaminomethylsulfonic acid (GAMS) has been reported to act as an antagonist of both AMPA and kainate receptors (Fig. 10.8) [186, 187], but with a preference for kainate receptors ($K_{\text{D}} \sim 50 \mu\text{M}$ [188]). Wilding and Huettnner [172], however, did not observe a selectivity for kainate receptors and found GAMS to have low potency in DRG neurons ($K_{\text{b}}=360 \mu\text{M}$). Alt et al. [97] also found GAMS to exhibit low potency with IC_{50} values in the range of 1–2 mM (corresponding to K_{b} values of $\sim 300 \mu\text{M}$) at recombinant human $\text{GLU}_{\text{K}5}$, $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$, and $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ receptors (Table 10.2). Although the activity observed at $\text{GLU}_{\text{K}5}$ -containing channels was weak, there appeared to be a degree of selectivity versus $\text{GLU}_{\text{K}6}$ since no antagonist effects were observed for GAMS at concentrations up to 3 mM at either $\text{GLU}_{\text{K}6}$ or $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}6}$ receptors.

10.3 ALLOSTERIC POTENTIATORS AND ANTAGONISTS

10.3.1 NMDA Receptor Allosteric Potentiators

Agonists at the glycine binding site: In an initial series of studies, Johnson and Ascher [36] showed that glycine (Fig. 10.9) was an important coagonist at the NMDA receptor and that this activity could be mimicked by some other neutral amino acids including D-serine (Fig. 10.9) and D-alanine. Careful work by Kleckner and

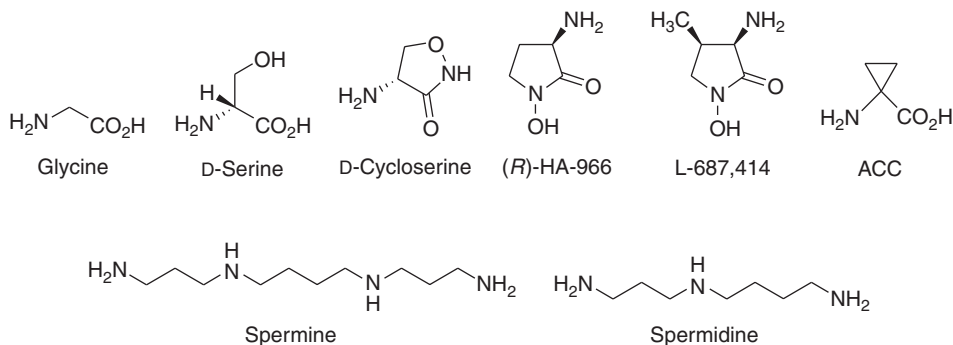


Figure 10.9 NMDA receptor allosteric potentiators.

Dingledine [37] demonstrated that occupation of this glycine site was a necessary requirement for NMDA receptor activation. Nanomolar concentrations of glycine appeared to be sufficient to enable activation by glutamate or NMDA, and these were thought to be provided by the endogenous levels of extracellular glycine. Indeed initial studies in vivo and in vitro suggested that this glycine site was fully occupied and addition of exogenous glycine or D-serine did not enhance NMDA receptor function. More recent studies have, however, suggested that this is not the case, and so potentially positive and negative modulation of NMDA receptor efficiency via this glycine site appears to be feasible (see [189]).

Following the initial discovery of this action of glycine, several analogs of glycine were found to be active with varying degrees of partial agonism. Among these D-cycloserine (Fig. 10.9) is a weak partial agonist but nevertheless has apparently some benefit in schizophrenia patients, as do massive doses of glycine and D-serine (see [35]). One of the less efficacious partial agonists is HA-966 (Fig. 10.9), which acts as a glycine site antagonist in vivo [190], a finding which explains its previously described NMDA antagonism [22]. Partial agonist analogs of HA-966 that are 10 times more potent than the parent compound have been developed as functional antagonists (e.g., L-687,414; Fig. 10.9) [191].

Of the newly discovered fuller agonists, aminocyclopropylcarboxylic acid (ACC) [192] is the most potent with an affinity an order of magnitude higher than that of glycine and D-serine. However, D-serine remains a useful tool for this site since unlike glycine it is not an agonist at the strychnine-sensitive inhibitory receptor and less subject to uptake by neutral amino acid transporter systems.

Positive allosteric modulators of NMDA receptor function: Ransom and Stec [38] described the NMDA potentiating (and inhibiting) effects of polyamines such as spermine and spermidine. There appear to be two facets to this potentiation [8, 193]: first, an increase in affinity of the GLU_{N1} subunit for glycine and, second, a glycine-independent potentiation of NMDA receptors containing GLU_{N2B} subunits and dependent on the splice variant of GLU_{N1} lacking exon 5 [194, 195]. The action of these positively charged polyamines appears to be mimicked by certain aminoglycoside antibiotics [196] and by magnesium ions [197, 198].

Interestingly, steroid hormones, more widely recognized for enhancing GABA function, also potentiate NMDA receptor function. This appears to be subtype selective, inasmuch as pregnenolone enhances heteromers containing the GLU_{N2A} and GLU_{N2B} subunits but inhibits those containing GLU_{N2C} and GLU_{N2D} subunits [199]. This NMDA potentiating effect of polyamines can be reversed by the peptide conantokin-G, derived from *Conus* snails [200]. Structure–activity studies with peptide fragments and analogs from conantokin-G have elucidated molecules that potentiate [201] NMDA receptor function or act as polyamine antagonists [202].

10.3.2 NMDA Receptor Allosteric Antagonists

Competitive glycine site antagonists: The first functional glycine site antagonists reported were HA-966 (Fig. 10.9) [190] and kynurenic acid [203]. It was subsequently discovered that these two compounds act fundamentally differently; that is, HA-966 is a weak partial agonist whereas kynurenic acid is a full antagonist [204, 205]. Structure–activity studies of both compounds have produced more potent and selective analogs.

The (*R*)-(+) enantiomer of HA-966 is the active agent. One of the most potent of the HA-966 analogs yet developed is L-687,414 with affinity in the low-micromolar range [206]. This potentially useful pharmacological agent crosses the blood–brain barrier to provide functional antagonism *in vivo* as evidenced by its neuroprotective effect in focal ischemia [207] but interestingly did not block LTP *in vivo* as do classical NMDA antagonists [208]. Other partial agonists include cyclobutyl and cyclopropyl substituted glycines [192, 209, 210] as well as D-cycloserine [211], which, at higher doses, reverses the benefits in schizophrenia seen at lower doses (see [35]). This illustrates the difficulty of using partial agonists for *in vivo* studies, because at low concentrations they act as weak agonists but at higher concentrations will appear as antagonists.

Kynurenine acid is, by comparison, a full antagonist at the glycine site but is very nonspecific, with antagonist effects at other glutamate receptors. The usefulness of kynurenate analogs was, however, indicated by the observation that the 7-chloro and 5,7-dichloro derivatives (Fig. 10.10) were quickly shown to be more potent and selective glycine site antagonists [205, 212, 213]. Interestingly, the quinoxalinediones DNQX and CNQX [214] show weak glycine site antagonism [215], in addition to their more potent effects at the AMPA receptors. Kynurenates and quinoxalinediones, thus, formed the basis for the structure–activity studies for the development of full glycine site antagonists. Among the most potent of these are L-689,560 (Fig. 10.10) [216], L-701,324 (Fig. 10.9) [217], ACEA-1021 (Fig. 10.10) [218], GV150526 (Fig. 10.9) [219], and MRZ 2/576 (Fig. 10.9) [220]. In general, however, these compounds have poor bioavailability due to plasma protein binding (see [221]) but nevertheless have been shown to have typical NMDA antagonist profiles, for example, anticonvulsant, neuroprotective, and analgesic, but appear to have less psychotomimetic potential [191]. GV150526 was assessed in two large phase III trials for stroke; however, no positive outcome was seen. There remain questions over the brain bioavailability of this compound and the length of the time to treatment of the stroke patients.

Channel blocking NMDA antagonists: Magnesium ions are the physiological blockers of the NMDA channel [18], controlling channel conductance in a voltage-dependent manner [222, 223] such that depolarization from resting potential increases inward current in the presence of an NMDA receptor agonist. This “positive-feedback” phenomenon is dependent on the normal levels of extracellular magnesium and underlies the characteristic electrophysiological and pathological properties of NMDA receptor activation. Depolarization relieves the magnesium block and allows cationic current including calcium via the NMDA channel in circumstances such as repeated synaptic activation via afferent input or continuous activation via high levels of extracellular glutamate. Interestingly, the block by magnesium ions at a site deep within the channel is greater on complexes containing GLU_{N2A}/GLU_{N2B} subunits than those containing GLU_{N2C}/GLU_{N2D} [46]. Other divalent cations vary in their ability to permeate or block the NMDA receptor channel [224].

Ketamine and phencyclidine (Fig. 10.10) were discovered as NMDA antagonists [32] as part of a study into the central mechanisms of short-acting anaesthetics [225]. Ketamine was also shown to block the “wind-up” phenomenon of neuropathic pain which in part explains its analgesic properties [226]. Block of the NMDA receptor occurred, however, at doses that were considerably less than those needed for

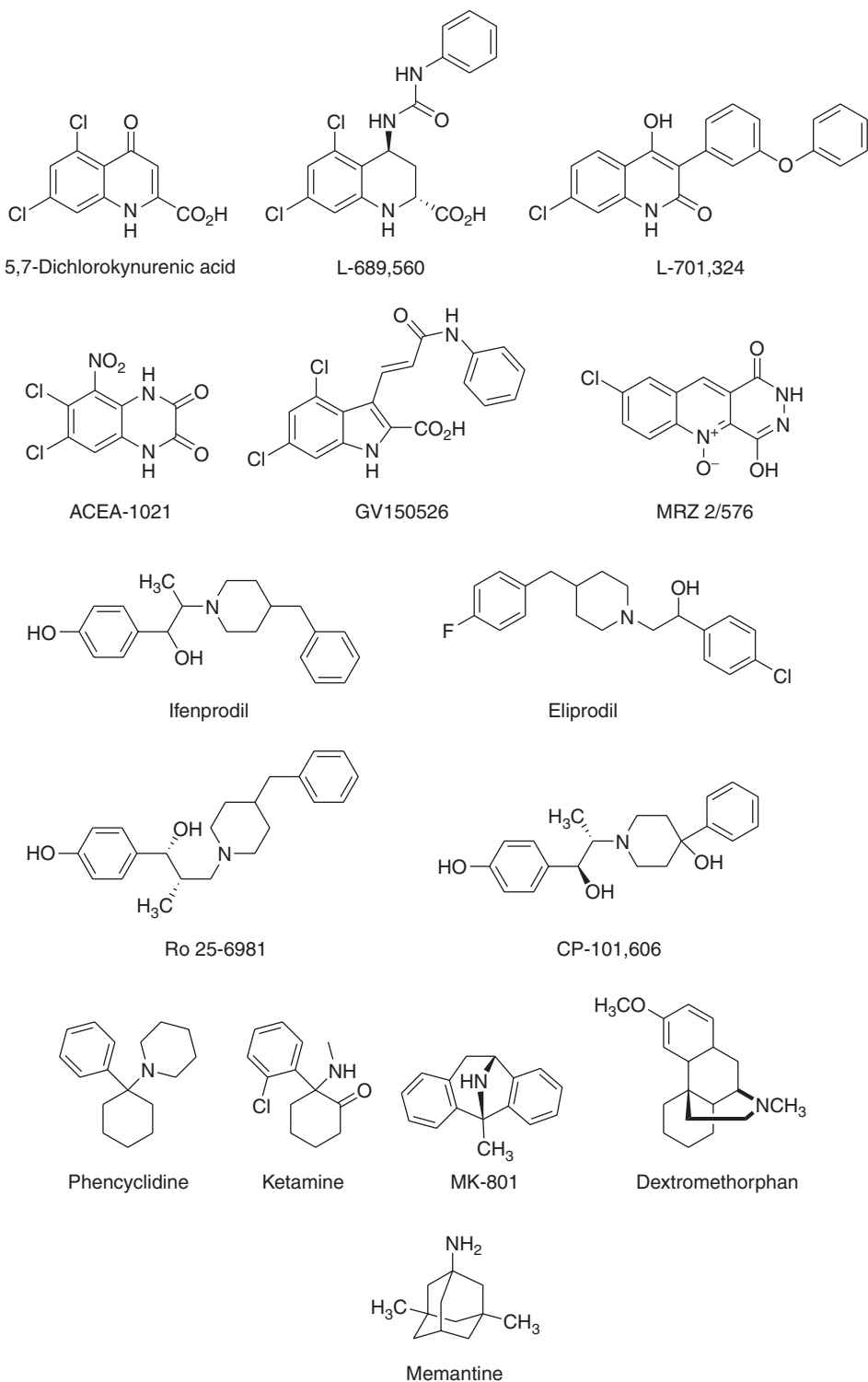


Figure 10.10 NMDA receptor allosteric antagonists.

anaesthesia and which were rather similar to those inducing “psychotomimetic-like” effects in laboratory animals (see [227, 228]). It quickly became apparent that many other drugs inducing psychotomimetic behavior in humans and/or generalization to phencyclidine in animal studies shared NMDA antagonistic properties [33]. The list includes the sigma opiates, SKF10,047, cyclazocine, and pentazocine [229], dioxalones [230], the morphinans dextromethorphan and dextrorphan (Fig. 10.10) [231], and the dibenzocycloalkenimine MK-801 (Fig. 10.10) [232]. All these channel blocking compounds display voltage and use dependence; that is, they need activation of the receptor channel complex for both access to and dissociation from the NMDA channel and, where examined, produce schizophrenomimetic effects in humans [233–236] (see [35]). MK-801 is the most potent and selective and is used as a standard in many experimental assays. It is, however, atypical in that it has a long duration of action, in part due to its slow off time even in the presence of agonist and is therefore likely to have profound and long-lasting psychotomimetic effects in humans. At the other end of the spectrum is memantine (Fig. 10.10), which has fast “on-and-off” kinetics and a reduced tendency to psychotomimetic effects [237].

In addition to such pharmacodynamic differences, their good bioavailability, and the range of pharmacokinetic properties, several channel blocking compounds offer useful characteristics to preclinical and clinical researchers. Thus brief reversible or long-duration block of NMDA receptors can be achieved with ketamine or MK-801, respectively. Ketamine and dextromethorphan, approved for use in humans, have been used for exploring the effects of NMDA blockade in normal volunteers and psychiatric patients (see [35]). These allosteric NMDA antagonists mimic the symptoms of schizophrenia in humans, and hence enhancing NMDA receptor-mediated synaptic transmission, directly or indirectly, becomes an obvious potential strategy for this psychiatric disease [34].

Ketamine has been in the clinic as an analgesic anaesthetic since the 1960s [233], and now some 40 years later memantine has been approved for the symptomatological treatment of Alzheimer’s dementia. Dextromethorphan, which is metabolized to the more potent NMDA antagonist dextrorphan, has been used as a cough suppressant, but whether NMDA antagonism plays a part in this clinical use is not known.

Newer and more potent channel blocking NMDA antagonists, for example, gavestinel [238] and aptigenel (Cerestat) [239], have entered stroke trials, but the clinical outcome has not been encouraging and the trials have been abandoned. Despite the site of action within the channel, there have been reports of selectivity toward GLU_{N2C} subunit-containing receptors for memantine (see [237]) and for dextromethorphan [240] and of slower kinetics for MK-801 at GLU_{N2C} -containing receptors [240].

Noncompetitive NMDA receptor antagonists: Polyamines such spermine and spermidine (Fig. 10.9) appear to be natural modulators of NMDA receptor function [38]. In addition to the potentiating effects (see above), they also produce a glycine-independent NMDA antagonism, probably via several sites [241, 242]. To some extent their action is mimicked by zinc, which displays a high-affinity voltage-independent inhibition, except that this cation shows strong selectivity for the GLU_{N2A} subunits over GLU_{N2B} with less potency at the other GLUN subtypes [243].

Ifenprodil (Fig. 10.10), a phenylethanolamine, is a potent noncompetitive NMDA antagonist [244], also glycine independent but with a strong preference for GLU_{N2B} -containing receptors over GLU_{N2A} and again with little or no activity at GLU_{N2C} / GLU_{N2D} [245, 246]. Unfortunately, the utility of ifenprodil is limited by its action, at sigma receptors [247] and at voltage-dependent calcium channels [248] as is that of the close analog eliprodil (Fig. 10.10) [249]. Other analogs, for example, Ro25-6981 (Fig. 10.10) [250] and CP101,606 (Fig. 10.10) [251], show similar selectivity for GLU_{N2B} but with reduced sigma and calcium channel properties. Some of these compounds have adverse effects on cardiac excitability and/or are metabolized rapidly by the liver.

It is hoped that later compounds such as benzamides [252], benzimidazoles [253], and isoquinolin-pyrimidines [254] are free of these and other disadvantages. These GLU_{N2B} -selective compounds, which act at the N-terminal domain, appear to have the beneficial properties of anticonvulsant, neuroprotective, analgesic, and so on, without the psychotomimetic downside and may even enhance some forms of memory [221, 255, 256]. This may be viewed as somewhat surprising since this subunit is widely expressed in the forebrain including the hippocampus [39, 45] and the GLU_{N2B} subunits appear to be involved in synaptic plasticity [257, 258]. On the other hand, there may be a predominance of the GLU_{N2B} subunit in extrasynaptic NMDA receptors [259, 260], for example, those activated by spillover as may occur in seizure activity, by high extracellular levels of glutamate as in ischemia, or high-frequency inputs as may occur in some pain states. Indeed, such extrasynaptic NMDA receptors appear to play a crucial role in some forms of neuronal cell death [261]. If GLU_{N2B} -containing receptors are less involved in normal synaptic processing of sensory information, then antagonists selective for these subunits may be expected to have less perceptual disturbances than nonselective compounds.

10.3.3 AMPA Receptor Allosteric Potentiators

Pyrrolidinones and benzamides: Several classes of positive allosteric modulators of AMPA receptor have been identified which have no effect on AMPA receptor function alone but in the presence of agonist binding can markedly enhance ion flux through the channel. The effects of several modulators from distinct chemical classes on the magnitude of the inward current evoked by application of AMPA are presented in Figure 10.11. Note the marked differences in the potency and efficacy of these compounds. One of the first modulators to be described was the pyrrolidinone 1-(4-methoxybenzoyl)-2-pyrrolidinone (aniracetam), which was shown to selectively potentiate quisqualate- and AMPA-evoked currents (but not kainite- or NMDA-evoked responses) in *Xenopus* oocytes as well facilitate glutamatergic synaptic transmission in electrophysiological recordings from hippocampal slices (Fig. 10.11) [262]. A structurally related series of benzamide compounds, also known as “AMPAkines,” including 1-(quinoxalin-6-ylcarbonyl)-piperidine (CX516), 1-(1,4-benzodioxan-6-ylcarbonyl)piperidine (CX546), and 2*H*,3*H*,6*aH*-pyrrolidino (2'',1''-3',2')1,3-oxazino(6',5'-5,4)benzo[*e*]1,4-dioxan-10-one (CX614), have been identified that also selectively enhance AMPA receptor-mediated synaptic responses in vitro and in vivo [263–269].

Benzothiadiazides: A structurally distinct series of benzothiadiazide modulators were identified following the observation that 7-chloro-3-methyl-2*H*-1,2,

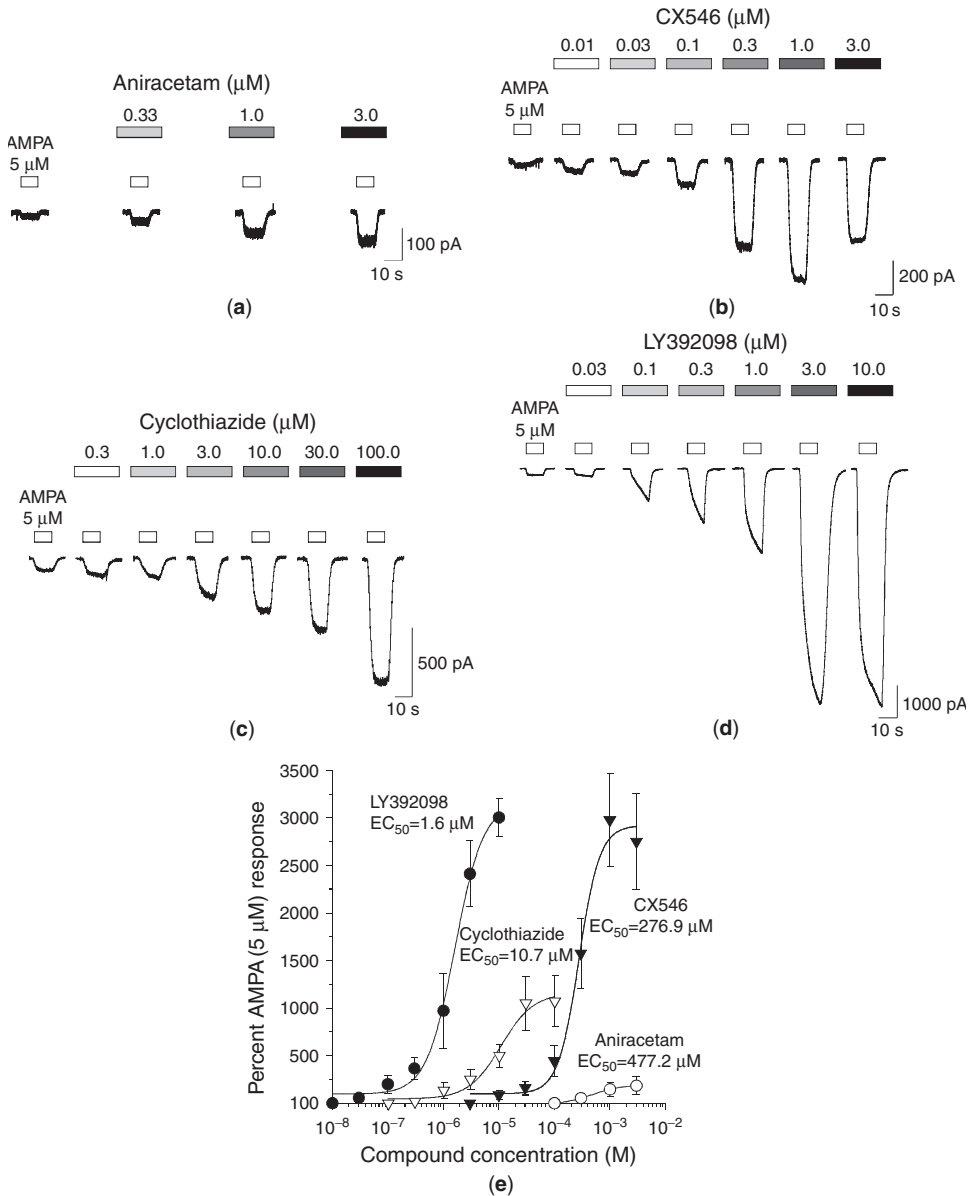


Figure 10.11 Concentration–response profile for aniracetam, cyclothiazide, CX546, and LY392098. Concentration–response profile for (a) aniracetam (0.3–3.0 mM, $n = 5$), (b) CX546 (0.01–3.0 mM, $n = 7$), (c) cyclothiazide (0.3–100 μM, $n = 9$), and (d) LY392098 (0.03–10.0 μM, $n = 8$) potentiation was assessed by measuring the responses of acutely isolated rat cortical neurons to 5 μM AMPA alone and in the presence of potentiator. Responses to each compound were from different neurons and reflect excerpts from a continuous chart recording. Each concentration of the potentiators was applied for approximately 20 s prior to application of AMPA. All compounds enhanced AMPA responses in concentration-dependent manner in the absence of any effects on their own. (e) Plot of average degree of potentiation as percent of response to AMPA (5 μM) alone for each concentration of each compound tested. Points represent mean \pm standard deviation (SD).

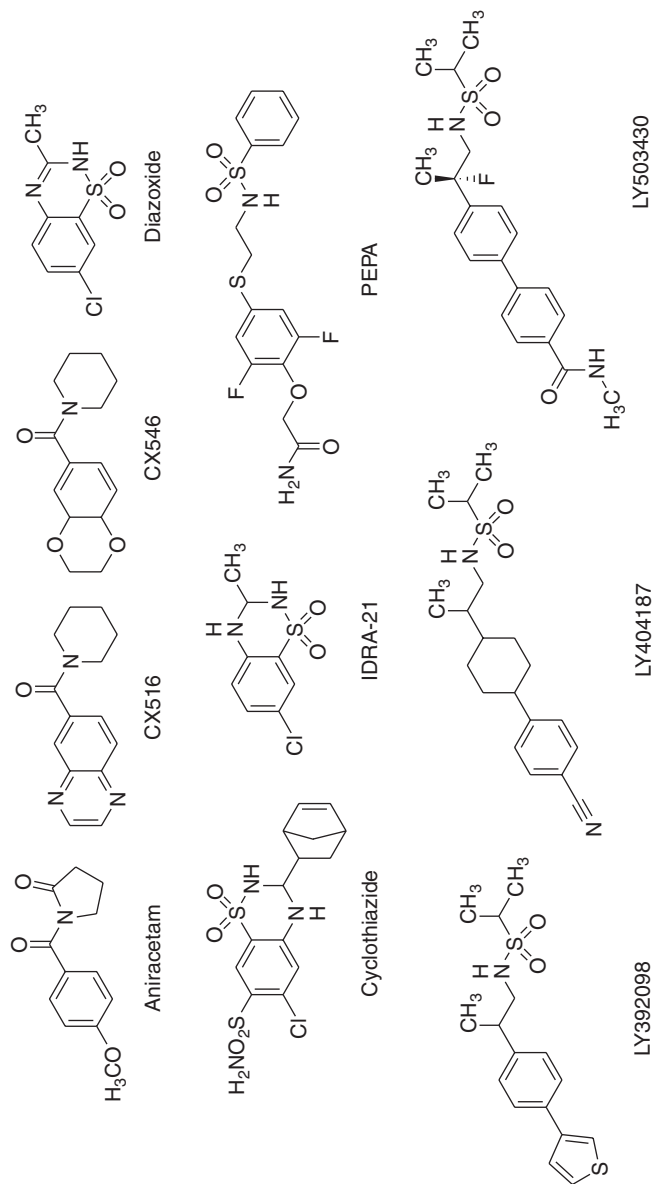


Figure 10.12 AMPA receptor allosteric potentiators.

4-benzothiadiazine 1,1-dioxide (diazoxide) selectively augmented quisqualate currents and enhanced excitatory postsynaptic currents in recordings from cultured hippocampal neurons [270]. Subsequent studies with a structurally related compound, 6-chloro-3,4-dihydro-3-(5-norbornen-2-yl)-2*H*-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide (cyclothiazide), demonstrated that AMPA receptor potentiation was associated with long bursts of glutamate-induced channel openings [271]. Other compounds from this class of modulators include 7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (IDRA-21) and (4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA) [272, 273].

Biarylpropylsulfonamides: More recently, a series of biarylpropylsulfonamides have been reported, including (*R,S*)-*N*-2-(4-(3-thienyl)phenyl)propyl-2-propanesulfonamide (LY392098), *N*-2-[4-(4-cyanophenyl)phenyl]propyl 2-propanesulfonamide (LY404187), *N*-2-(4-(*N*-benzamido)phenyl) propyl-2-propanesulfonamide (LY395153), and (*R*)-4'-[1-fluoro-1-methyl-2-(propane-2-sulfonylamino)-ethyl]-biphenyl-4-carboxylic acid methylamide (LY503430) [274–278]. Similar to the functional consequences reported for other classes of positive allosteric modulators, the biarylpropylsulfonamides selectively enhance ion flux in recombinant homomeric receptors and native heteromeric receptors and potentiate AMPA receptor-dependent synaptic responses in brain slices and in vivo [274, 279–281].

Apart from their structural differences, positive allosteric modulators can display marked preferences for modulating the flip-and-flop alternative splice variants of AMPA receptors. Cyclothiazide, LY404187, and LY503430 preferentially modulate homomeric receptors composed of the flip isoform of GLU_{A1–4} subunits [62, 278, 282]; whereas aniracetam and PEPA display greater activity at receptors expressing flop splice-variants [273, 282]. Site-directed mutagenic studies have indicated that splice-variant selectivity depends in large part on the identity of a single amino acid within the flip/flop extracellular domain. The presence of a serine or an asparagine residue at the position corresponding to 775 in full-length human GLU_{A2} subunit is critical for determining sensitivity of cyclothiazide or LY404187 to flip or flop isoforms, respectively [281, 282]. Similarly, the preferential flop receptor sensitivity to aniracetam is dependent on this residue [282].

Positive allosteric modulators of AMPA receptors also can have distinct biophysical mechanisms of action by preferentially affecting deactivation or desensitization of the receptor. For example, cyclothiazide and PEPA attenuate desensitization with minimal effects on deactivation, whereas, aniracetam slows deactivation with less effect on desensitization [273, 282]. Other molecules such as CX614 can affect both processes [267, 283]. Insights into the molecular determinants for modulation of deactivation and desensitization of AMPA receptors have come from studies of cocrystal structures of the GLU_{A2} LBC in complex with cyclothiazide (desensitization) or aniracetam and CX614 (deactivation) [64, 283]. For cyclothiazide, two molecules bind in the dimer interface between adjacent LBCs such that the long axis of each compound spans the interface between the upper and lower domains of the LBC. In so doing, cyclothiazide stabilizes the dimer interface, precluding rearrangement of the dimer and subsequent desensitization [64]. For aniracetam and CX614, only one molecule binds at the dimer interface and the long axes of the molecules are oriented approximately parallel to the interface. These modulators do not markedly infiltrate the interface between the upper and lower domains of the LBC but rather bind adjacent to the “hinge” of the clamshell LBC. These molecules exert their

actions by stabilizing the closed-cleft, glutamate-bound conformation of the LBC and thereby attenuate unbinding of glutamate and deactivation of the receptor [283]. It should be noted that although modulators can preferentially affect one biophysical process over another, they are not completely selective (e.g., aniracetam has some effect on desensitization). The lack of exclusivity in modulation of deactivation and desensitization most likely results from the fact that some overlap exists in the binding sites of the modulators [283].

An accumulating body of evidence suggests that positive allosteric modulation of AMPA receptors may have therapeutic implications in a variety of neurological and psychiatric disorders. Early clinical studies with the pyrrolidinone compounds piracetam and aniracetam showed modest results in treating cognitive deficits associated with several neurological diseases, including Parkinson's disease, Alzheimer's disease, and schizophrenia [284–286], prompting their informal classification as “nootropic agents” (i.e., compounds that facilitate learning and memory). As described above, AMPA receptor modulators enhance glutamatergic synaptic activity due to the direct potentiation of AMPA receptor function. In addition, these compounds can indirectly recruit voltage-dependent calcium channels and NMDA receptor activity [279]. Enhanced calcium influx through NMDA receptors is known to be a critical step in initiating long-term modifications in synaptic function (e.g., LTP). These modifications in synaptic function may be substrates for certain forms of memory encoding [269]. Consistent with a recruitment of NMDA receptor activity, AMPA receptor modulators have been shown to facilitate the induction of LTP as well as improve performance in animal models of cognitive function requiring different mnemonic processes [263, 264, 287–290]. Along with their nootropic actions, these positive AMPA receptor modulators have been shown to be active in other models of disease. For example, piracetam is efficacious in several models of anxiety, including the social interaction test, elevated plus maze, and Vogel's conflict test [291, 292]. CX516 (and structural analogs) has been demonstrated to suppress hyperactivity in animal models of attention-deficit hyperactivity disorder (ADHD) and positive symptoms of schizophrenia [293, 294].

In addition to enhancing glutamatergic synaptic transmission, AMPA receptor modulators have been shown to increase levels of the neurotrophin brain-derived neurotrophic factor (BDNF) in neuronal culture [295, 296] and in vivo [278, 297]. Increases in BDNF levels have been associated with the activity of several classes of antidepressant therapies and with development, growth, and maintenance of neurons, which may be a critical component of antidepressant efficacy [298]. BDNF mRNA has been shown to be downregulated in rats tested in animal models of behavioral despair, including restraint stress and the forced-swim test, and this reduced expression can be reversed by antidepressant treatment [299, 300]. Because of their ability to enhance BDNF expression, allosteric modulation of AMPA receptors has been proposed as a novel strategy for the treatment of depression [301]. Indeed, administration of the biarylpropylsulfonamide LY392098 reduced immobility in both mice and rat forced-swim tests, a model of behavioral despair that can be used to detect antidepressant compounds [302]. In addition, the effects of LY392098 could be blocked by the selective AMPA receptor antagonist LY300168, indicating that the effect of the potentiator was mediated through AMPA receptors [302]. Finally, the neurotrophic effects of AMPA receptor potentiators have been extended experimentally to models of Parkinson's disease. In these studies, administration of

LY503430 was shown to reverse the neurochemical and behavioral deficits associated with dopamine-depleting brain lesions as well as produce modest enhancements in BDNF expression in the dopamine cell body region of the substantia nigra [303]. While the precise mechanisms through which positive allosteric modulators of AMPA receptors exert their beneficial effects in models of antidepressant activity and Parkinson's disease remain to be elucidated, these results suggest that positive modulators may be efficacious in a wide range of therapeutic targets.

10.3.4 AMPA Receptor Allosteric Antagonists

2,3-Benzodiazepines: The first examples of negative allosteric modulators of AMPA receptors came from a series of 2,3-benzodiazepines typified by 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466) and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-3-methylcarbamoyl-2,3-benzodiazepine (GYKI 53784) (also known as LY303070; racemate LY300168) (Fig. 10.13) [304, 305]. In contrast to the 1,4-benzodiazepines, the 2,3-benzodiazepines have no affinity for the GABA_A receptor but block native and recombinant AMPA receptors with low-micromolar potency in a noncompetitive manner [175, 306–309]. In addition, these compounds have very weak potency at kainate receptors and as such have become an important tool for distinguishing between AMPA and kainate receptor-mediated responses.

Quinazolinones: Another class of selective negative allosteric modulators of AMPA receptors from a quinazolinone series has been reported and includes 3-(2-chlorophenyl)-2-[2-(6-diethylaminomethyl-pyridin-2-yl)-vinyl]-6-fluoro-3*H*-quinazolin-4-one (CP-465,022) and 2-{2-[3-(2-chloro-phenyl)-6-fluoro-4-oxo-3,4-dihydro-quinazolin-2-yl]-vinyl}-nicotino nitrile (CP-526,427) [310–313].

Although crystallographic analyses of the AMPA receptor LBC in conjunction with negative allosteric modulators have not been performed, pharmacological and receptor binding experiments have ruled out some possible binding sites. For example, [³H]CP-526,427 specific binding to rat forebrain membranes was not significantly affected by the orthosteric agonists glutamate, AMPA, and kainate or the orthosteric antagonist YM-90K, confirming the noncompetitive nature of CP-526,427 [311]. However, [³H]CP-526,427 binding was inhibited by the noncompetitive 2,3-benzodiazepine antagonist GYKI52466, suggesting overlap in the binding sites of these negative allosteric modulators. Similar to the results with orthosteric compounds, the positive allosteric modulators cyclothiazide, 1-BCP, or CX-516 at concentrations that

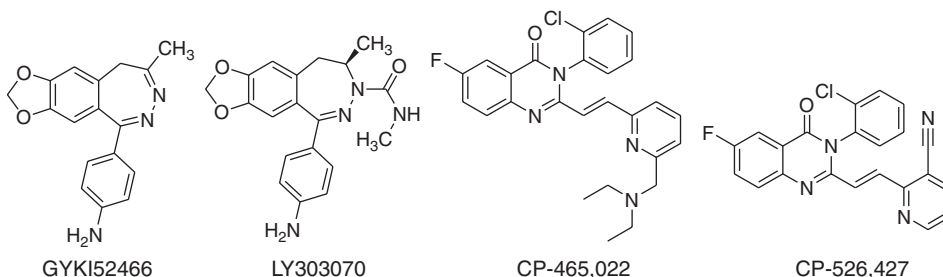


Figure 10.13 AMPA receptor allosteric antagonists.

are known to enhance AMPA receptor-mediated currents were ineffective in blocking [^3H]CP-526,427 specific binding [311]. Consistent with these latter data, studies have shown that while mutation of the serine 775 residue in the flip/flop domain to glutamine (corresponding residue in kainate receptors) eliminates the activity of cyclothiazide, this substitution has no effect on the antagonism of the 2,3-benzodiazepine GYKI53655 [314]. Both of these observations suggest that the binding sites of negative and positive allosteric modulators of AMPA receptors are distinct.

The possibility that negative allosteric modulators of AMPA receptors may provide a novel anticonvulsant therapy has been explored extensively in recent years. Several of the 2,3-benzodiazepines [e.g., GYKI52466, LY300164 (Talampanel)] have potent anticonvulsant activity in a broad range of in vivo epilepsy models (for review, see [315]). In addition, LY300164 can potentiate the anticonvulsant action of known antiepileptic medications, including valproate, carbamazepine, and phenobarbital [316]. A potential limitation of negative allosteric modulators as suggested by the effects of competitive antagonists is a narrow therapeutic window due to adverse or undesirable side effects. For example, NBQX blockade of AMPA receptors is known to produce ataxia and can impair learning and memory in rats [317]. In contrast, GYKI52466 does not block the induction of LTP in hippocampal brain slices [318] and LY300164 does not exacerbate the ataxia and cognitive impairment of traditional antiepileptics [316]. More importantly, in a recent clinical trial, LY300164 significantly reduced seizure frequency in patients with partial refractory seizures in the absence of cognitive impairment (although ataxia was observed in 26% of the subjects) [319]. While no pivotal clinical trials have been completed, these results are encouraging and suggest that negative allosteric modulators may be efficacious as anticonvulsants. Finally, evidence suggests that several other diseases associated with elevated brain glutamate concentrations also may benefit from AMPA receptor negative modulators, particularly because of their ability to block glutamatergic neurotransmission without having to surmount the high levels of endogenous glutamate. Negative modulators have been shown to be beneficial in studies using animal models of ischemia stroke, Parkinson's disease, multiple sclerosis, and persistent pain (for review, see [315]).

10.3.5 Kainate Receptor Allosteric Potentiators

Concanavalin A: As previously discussed, kainate receptors exhibit rapid desensitization upon continued agonist application. Therefore many studies of kainate receptors, especially those involving transfected stable cell lines, use concanavalin A, a lectin from jack bean, to block agonist desensitization. The exact method of action of concanavalin A remains to be elucidated. However, it is known that concanavalin A binds to several glycosylation sites on the extracellular domain of the receptor [320]. Concanavalin A has been reported to increase agonist affinity at kainate receptors. Paternain et al. [321] reported that the EC_{50} for kainate at rat $\text{GLU}_{\text{K}6}$ following treatment with concanavalin A was 25-fold lower than the value from untreated receptors. Jones et al. [322] reported that the EC_{50} for SYM2081 at rat $\text{GLU}_{\text{K}6}$ was also reduced by concanavalin A treatment. However, other studies have demonstrated only a small effect or no effect on agonist EC_{50} values at kainate receptors comprised of $\text{GLU}_{\text{K}5}$ [179, 323]. $\text{GLU}_{\text{K}7}$ receptors are insensitive to concanavalin A, a feature which has limited their study.

10.3.6 Kainate Receptor Allosteric Antagonists

NS3763: Recently, the first allosteric antagonists of kainate receptors have been described. 5-Carboxyl-2,4-di-benzoamido-benzoic acid (NS3763) was shown to inhibit glutamate- or domoate-evoked currents in HEK293 cells recombinantly expressing human kainate receptors (Fig. 10.14) [160]. NS3763 was found to insurmountably antagonize both domoate and glutamate. The noncompetitive nature of NS3763 was further evidenced by its inability to inhibit [^3H]ATPA binding to $\text{GLU}_{\text{K}5}$ receptors. NS3763 was found to be selective for the $\text{GLU}_{\text{K}5}$ receptor subtype ($\text{IC}_{50} = 1.6 \mu\text{M}$) versus $\text{GLU}_{\text{K}6}$, AMPA, or NMDA receptors ($\text{IC}_{50} > 30 \mu\text{M}$). Perhaps the most interesting property of NS3763 is that, unlike other $\text{GLU}_{\text{K}5}$ -selective antagonists such as the decahydroisoquinolines or willardiine derivatives (see above), it has been reported to be selective for homomeric $\text{GLU}_{\text{K}5}$ versus heteromeric $\text{GLU}_{\text{K}2}/\text{GLU}_{\text{K}5}$ or $\text{GLU}_{\text{K}5}/\text{GLU}_{\text{K}6}$ receptors, making this compound a potentially useful tool for examining the subunit composition of native kainate receptors [183a].

2-Arylureidobenzoic acids: A series of 2-arylureidobenzoic acids have also been described as noncompetitive $\text{GLU}_{\text{K}5}$ -selective antagonists [324, 325]. Like NS3763, these compounds are potent antagonists at $\text{GLU}_{\text{K}5}$ channels. 4-Chloro-2-(3-naphthalen-2-yl-ureido)-benzoic acid (2-arylureidobenzoic acid) and 2-[3-(3-bromophenyl)ureido]-4-chlorobenzenesulfonic acid are the most potent compounds from this series described to date, blocking calcium influx evoked by $2 \mu\text{M}$ domoate at recombinant human $\text{GLU}_{\text{K}5}$ with IC_{50} values of 1.2 and $1.5 \mu\text{M}$, respectively [325]. These compounds are selective for $\text{GLU}_{\text{K}5}$ versus $\text{GLU}_{\text{K}6}$, blocking domoate ($0.2 \mu\text{M}$)-evoked calcium influx at recombinant human $\text{GLU}_{\text{K}6}$ receptors with reported IC_{50} values of $62 \mu\text{M}$ for 2-arylureidobenzoic acid and $37 \mu\text{M}$ for 2-[3-(3-bromophenyl)ureido]-4-chlorobenzenesulfonic acid. Neither compound showed significant activity at AMPA receptors, with $\text{IC}_{50} > 100 \mu\text{M}$ for blocking glutamate ($25 \mu\text{M}$)-evoked calcium influx at recombinant human $\text{GLU}_{\text{A}1-4}$ [325]. Like NS3763, these compounds do not inhibit the binding of [^3H]ATPA to recombinant human $\text{GLU}_{\text{K}5}$ receptors.

10.4 FUTURE PERSPECTIVES

The cloning and expression of recombinant proteins for NMDA, AMPA and kainate receptors have facilitated the identification of many potent and selective compounds. The use of these agents as pharmacological tools both in vitro and in vivo has enabled recent advances in our understanding of the role of ionotropic glutamate receptors in the central nervous system. More recently, agents have been discovered which show increased potency and improved selectivity for subtypes within each of the three classes of receptor. X-ray crystallographic data for each receptor have

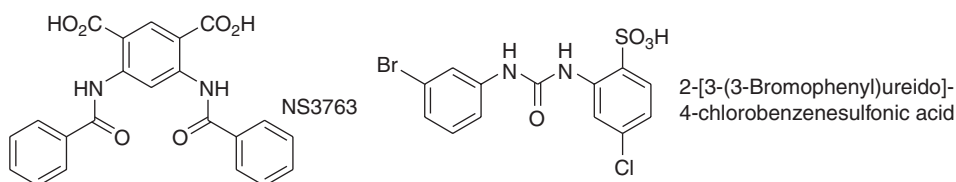


Figure 10.14 KA receptor allosteric antagonists.

allowed an insight into the topographical structure of the ion channel and the potential binding interactions of ligands to specific domains. As new agents emerge that show high-affinity interactions with either orthosteric or allosteric sites, we may expect to increase our knowledge of chemical features that either enhance affinity or confer greater selectivity. A key area of future research will be to better understand the specific subunit composition that comprises a native ion channel receptor. Additionally, it will be important to explore the influence of such heterogeneity on the behavior of pharmacological agents. The critical role of ionotropic glutamate receptors in synaptic transmission makes them ideal targets for therapeutic intervention. An emergent area of research is work on how glutamate receptors are altered in disease (e.g., distribution, phosphorylation state, subunit composition). It may be necessary for us to be able to model such changes in order to be successful in specific pharmacological intervention.

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11

METABOTROPIC GLUTAMATE RECEPTORS

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11.1	Background/Receptor Classification	421
11.2	Orthosteric Pharmacological Agents	423
11.2.1	Group I Selective Orthosteric Agonists	423
11.2.2	Group I Selective Orthosteric Antagonists	424
11.2.3	Group II Selective Orthosteric Agonists	426
11.2.4	Group II Selective Orthosteric Antagonists	429
11.2.5	Group III Selective Orthosteric Agonists	431
11.2.6	Group III Selective Orthosteric Antagonists	433
11.3	Subtype Selective Allosteric Potentiators and Antagonists	435
11.3.1	mGlu1 Allosteric Potentiators	435
11.3.2	mGlu1 Allosteric Antagonists	436
11.3.3	mGlu2 Allosteric Potentiators	437
11.3.4	mGlu2/3 Allosteric Antagonists	439
11.3.5	mGlu4 Allosteric Potentiators	440
11.3.6	mGlu5 Allosteric Potentiators	441
11.3.7	mGlu5 Allosteric Antagonists	443
11.3.8	mGlu7 Allosteric Antagonists	445
11.4	Future Perspectives	445
	References	446

11.1 BACKGROUND/RECEPTOR CLASSIFICATION

Metabotropic glutamate (mGlu) receptors are a relatively novel heterogenous family of G-protein-coupled receptor proteins which function to modulate glutamate transmission via presynaptic, postsynaptic, and glial mechanisms. Currently, eight subtypes of mGlu receptors (mGlu1–8) have been cloned, and their structure and function have been described in various earlier reviews [1–6]. Briefly, group I mGlu receptors are positively coupled to phospholipase C and include mGlu1 and mGlu5

(and their splice variants). Both group II mGlu receptors (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8) are coupled via G_i to inhibition of adenylate cyclase. mGlu receptors have been divided into these three groups based on (1) structural homology of about 70% within groups and about 40% between groups, (2) similar transduction mechanisms or G-protein coupling, and (3) shared pharmacological properties within a group [7, 8]. Each receptor subtype and in some cases alternatively spliced versions are uniquely distributed, though many synapses likely express multiple subtypes, suggesting some redundancy of function (see Fig. 11.1).

Due to the high structural homology within mGlu groups, many current agents, particularly orthosteric (or glutamate binding site) agonist and antagonist ligands, are selective for mGlu receptor groups (group I, II, and III) but they do not distinguish subtypes within a group (e.g., mGlu2 and mGlu3). The discovery of agents with subtype selectivity within an mGlu receptor group has now greatly progressed with the advent of library screening for functional antagonists and potentiators which generally act by an allosteric mechanism (9–11).

The literature on the effects of many known pharmacological agents across all eight mGlu receptor subtypes has been previously reviewed [1, 2, 8]. In this chapter, we review the pharmacology of both orthosteric and allosteric targets for mGlu receptors and briefly comment on their usefulness as pharmacological tools to drive new knowledge in this area.

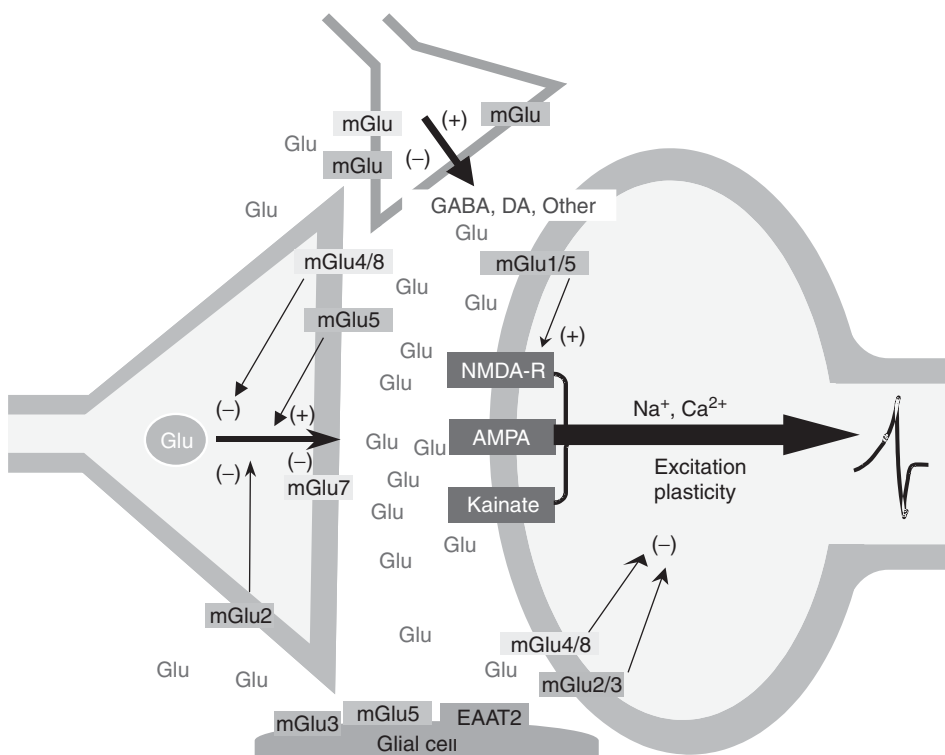


Figure 11.1 Glutamate synapse. (See color insert.)

11.2 ORTHOSTERIC PHARMACOLOGICAL AGENTS

11.2.1 Group I Selective Orthosteric Agonists

Quisqualate: Before the cloning of mGlu receptor subtypes in the early 1990s, mGlu receptors were generally characterized by the ability of glutamatergic acidic amino acid analogs to activate the phosphoinositide hydrolysis second-messenger system or subsequently increase intracellular calcium mobilization in a number of preparations, including cultured neurons, brain slices, cultured glia, and *Xenopus* oocytes injected with rat brain messenger ribonucleic acid (mRNA). The pharmacology of this response was clearly distinguished from known ionotropic glutamate receptor subtypes at that time [12, 13]. Quisqualate (Fig. 11.2) was the most potent mGlu receptor agonist in eliciting these metabotropic responses, with submicromolar potency in many tissues. Quisqualate also has high potency as an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor agonist, and this has generally precluded the use of quisqualate to study mGlu receptors in most preparations. Nevertheless, quisqualate has been useful as a group I selective agonist in recombinant systems and rat brain tissues. In particular, ^3H -quisqualate was used as a high-affinity ligand to study structure/function of cloned mGlu receptors in a recombinant system where its AMPA receptor activity would not interfere [14].

1*S*,3*R*-ACPD: The conformationally constrained cyclopentane derivative of glutamate, 1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD), gives rise to four different isomers; 1*S*,3*R*, 1*R*,3*S*, 1*S*,3*S*, and 1*R*,3*R* [12]. Racemic (\pm)*trans*-ACPD [or *cis*-ACPD according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature] was the first mGlu receptor selective agonist, shown to activate phosphoinositide hydrolysis in rat hippocampus at concentrations having no effects on ionotropic glutamate receptor ligand binding [15, 16]. Separation of (+)*trans*-ACPD into its 1*S*,3*R* and 1*R*,3*S* isomers demonstrated that 1*S*,3*R*-ACPD (Fig. 11.2) was the active component responsible for stimulation of phosphoinositide hydrolysis in rat brain tissue [17]. Also, consistent with its metabotropic glutamate receptor activity in rat brain slices, 1*S*,3*R*-ACPD (not 1*R*,3*S*-ACPD) was found to stimulate intracellular calcium mobilization in neurons [18]. However, 1*S*,3*R*-ACPD also acts as an agonist at multiple mGlu receptor subtypes, including mGlu1, mGlu2, mGlu3, mGlu4, mGlu5, mGlu6, and mGlu8 (see [8]). This non-subtype selective mGlu receptor agonist profile likely explains why a range of pharmacological actions have been reported for 1*S*,3*R*-ACPD (see [1, 3, 13]).

3,5-DHPG: (*R,S*)-3,5-Dihydroxyphenylglycine (3,5-DHPG; Fig. 11.2) was described as a more potent activator of rat mGlu1a receptors expressed in *Xenopus*

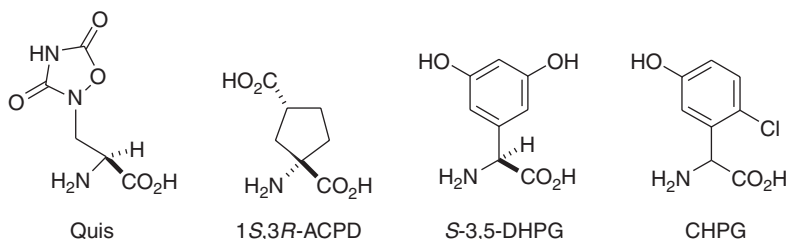


Figure 11.2 Group I selective orthosteric agonists.

oocytes than (\pm)*trans*-ACPD [19], when (\pm)*trans*-ACPD was one of the few known selective mGlu receptor agonists. In rat hippocampal CA3 pyramidal cells 3,5-DHPG, like ACPD, produced a slow excitation resulting from depression of inhibitory currents (I_{AHP} and I_{M}) [19]. This showed phenylglycines such as 3,5-DHPG could mimic the actions of more “glutamate-like” diacidic amino acids at mGlu receptors and led to the synthesis of many additional phenylglycines in the search to find more selective orthosteric agonists and antagonists [8, 20].

Subsequently, second-messenger studies in the rat hippocampus reported that 3,5-DHPG activated phosphoinositide hydrolysis but, unlike 1*S*,3*R*-ACPD, had no effect on forskolin-stimulated cAMP formation [21]. The activity of 3,5-DHPG on the phosphoinositide hydrolysis pathway in the rat hippocampus resided in the *S* isomer [22]. 3,5-DHPG exhibits about equal agonist potencies at mGlu1 and mGlu5 receptor subtypes but has no agonist or antagonist activities at recombinant rat and/or human group II (mGlu2 and mGlu3) or group III (mGlu4, 6, 7, or 8) mGlu receptors (see [23–26]).

The group I (mGlu1/5) selectivity of 3,5-DHPG has made this compound the most widely used for investigating the role of group I mGlu receptors in native tissues. In hippocampus and striatum 3,5-DHPG, although not directly exciting neurons, enhanced neuronal excitations by the potentiation of the ionotropic glutamate receptor agent *N*-methyl-D-aspartate (NMDA) [27, 28]. Central administration of 3,5-DHPG has been shown to lead to the induction of seizures and/or neurotoxicity [29–31] and produced pain-like states when administered centrally [32, 33] or peripherally [34] in animals. Studies such as these are consistent with the potential anticonvulsant, neuroprotectant, and analgesic actions of selective group I receptor antagonists in animals [35–37].

CHPG: (*R,S*)-2-Chloro-5-hydroxyphenylglycine (CHPG) is a *chloro*-substituted phenylglycine analog of 3HPG (see Fig. 11.2). CHPG has significant mGlu5 receptor activity (median effective concentration EC_{50} 750 μM) but no appreciable mGlu1 receptor activity at up to 10,000 μM [38], making it potentially useful for examining the relative role of mGlu5 versus mGlu1 receptors. Nevertheless, the low potency of CHPG and the more recent discovery of highly potent and selective mGlu5 receptor antagonists limit its usefulness to study mGlu5 receptor activation.

11.2.2 Group I Selective Orthosteric Antagonists

Before the 1990s the only selective antagonist for mGlu receptor responses, in this case phosphoinositide hydrolysis in rat brain tissues, was L-2-amino-3-phosphonopropanoate (L-AP3) (see [8, 12]). The search for more potent competitive mGlu receptor antagonists led to the recognition that various other phenylglycine derivatives possess antagonist, agonist, and even mixed agonist–antagonist activities across multiple subtypes of mGlu receptors (see [8, 39, 40]). In addition to certain recombinant receptors, many of these compounds were characterized in the neonatal spinal cord preparation [41, 42] where group I agonists (e.g., 1*S*,3*R*-ACPD, 3,5-DHPG) depolarize motoneurons, while group II selective agonists (e.g., 1*S*,3*S*-ACPD) and group III selective agonists (e.g., L-AP4) depress the monosynaptic component of the dorsal root-evoked ventral root potential (mDR-VRP).

(*S*)-4CPG: Substitution of the *para*-hydroxy group in 3,5-dihydroxyphenylglycine with a carboxy group, as in *S*-4-carboxy phenylglycine (*S*-4CPG; Fig. 11.3),

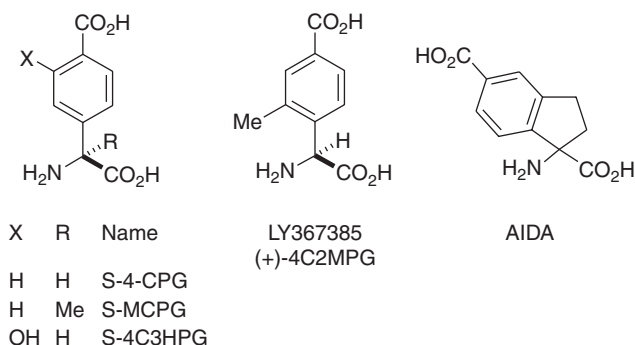


Figure 11.3 Group I selective orthosteric antagonists.

produced a mGlu1 selective competitive group I antagonist (median inhibitory concentration IC_{50} 10–100 μ M) with weak agonist activity at mGlu2 (EC_{50} \sim 500 μ M) but no activity at mGlu4 receptors [8, 43, 44].

(S)-MCPG: The incorporation of an α -methyl group into *S*-4CPG, as in *S*- α -methyl-4-carboxyphenylglycine [(*S*)-MCPG; Fig. 11.3] produced group I antagonist potency with about equivalent antagonist potency at mGlu2 receptors [8, 43–45], and no appreciable mGlu4 receptor activity. (*S*)-MCPG also had significant antagonist activity at mGlu8 receptors [26, 46, 47]. (*S*)-MCPG blocks the actions of L-AP4 in the neonatal spinal cord [47], suggesting that mGlu8 receptors mediate the depressant effects of L-AP4 in that preparation. (*S*)-MCPG became a very widely used pharmacological tool for probing the functions of mGlu receptors in cellular processes (see [40]). However, the discovery of more potent, selective, and systemically available non-phenylglycine antagonist compounds limits the current usefulness of (*S*)-MCPG.

(S)-4C3HPG: (*S*)-4C3HPG (Fig. 11.3) is an antagonist at mGlu1 receptors but is about equipotent as an agonist at mGlu2, with no appreciable activities at mGlu4 [43, 44]. Both group I antagonists and group II agonists have been shown to be anticonvulsant in certain animal models of epilepsy, and (*S*)-4C3HPG combines both of these activities in one molecule. Interestingly, Thomsen et al. [48] found that (*S*)-4C3HPG was active following intracerebral injection as an anticonvulsant against audiogenic seizures in DBA/2 mice. Thus, compounds with a group II agonist/group I antagonist profile might be more effective than either mGlu activity alone. This may also be the case for ischemia, as (*S*)-4C3HPG reduces infarct size following focal ischemia in rats [49], where group II mGlu2/3 receptor agonists per se are not active in rat stroke models [50].

AIDA: (*R,S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA; Fig. 11.3) is a reported selective antagonist of mGlu1 (pA_2 = 4.21), with weaker antagonist action at mGlu5, but was inactive at mGlu2 receptors [51]. The discovery of more potent and selective mGlu1 antagonists has limited the use of this compound. Nevertheless, target validation studies using centrally administered AIDA are consistent with other literature suggesting use of mGlu1 antagonists as analgesics [52], anxiolytics [53], and neuroprotectants [54, 55].

LY367385: ((+)-2-Methyl-4-carboxyphenylglycine (LY367385) is a micromolar potent and subtype selective competitive mGlu1 receptor antagonist [8, 56, 57].

LY367385 is not systemically active, but its relatively high water solubility and low micromolar potency have made it useful to explore mGlu1 receptor functions.

For example, the intracerebral injection of either LY367385 or AIDA was shown to be anticonvulsant in three different rodent models, including DBA/2 mice, lethargic mice, and genetically epilepsy prone rats [58]. This suggests a role for mGlu1 antagonists in the treatment of epilepsy. Likewise, LY367385 selectively reduced noxious excitatory responses in the rat thalamus [59], also suggesting a role for mGlu1 receptors in nociceptive processes of the brain. LY367385 is neuroprotectant in various models, including NMDA-induced excitotoxicity in vitro and in vivo, and intraventricular administration reduced hippocampal cell death in gerbils following transient global ischemia [57, 60].

11.2.3 Group II Selective Orthosteric Agonists

L-CCG-I: 2-(Carboxycyclopropyl)glycines (CCGs) are conformationally restricted glutamate analogs in which the proximal and distal carboxy groups can exist in either an extended or folded conformation. Studies with the isomers of CCG suggested that the extended conformation is preferred for mGlu receptor agonist potency and selectivity. In particular, L-CCG-I (Fig. 11.4) effectively activates multiple mGlu receptor subtypes, while having no effects on ionotropic glutamate receptors [8, 61–65]. As there are now compounds with much greater subgroup or subtype selectivity than L-CCG-I (see below), the usefulness of L-CCG-I as a pharmacological tool is fairly limited.

DCG-IV: Combining elements that led to the mGlu selective agonist L-CCG-I (extended glutamate conformation) and the potent NMDA agonist L-CCG-IV (folded glutamate conformation) resulted in the 2',3'-dicarboxycyclopropylglycine analog DCG-IV (Fig. 11.4) [63]. DCG-IV was described as a highly potent (nanomolar) and relatively selective agonist for group II mGlu receptors [64, 66]. However, at higher micromolar concentrations it is an antagonist at group I and group III mGlu receptor subtypes [64] and has been reported to activate NMDA receptors in native tissues [67–69]. ³H-DCG-IV has shown to bind to CHO cell membranes expressing recombinant rat mGlu2 receptors with relatively high affinity

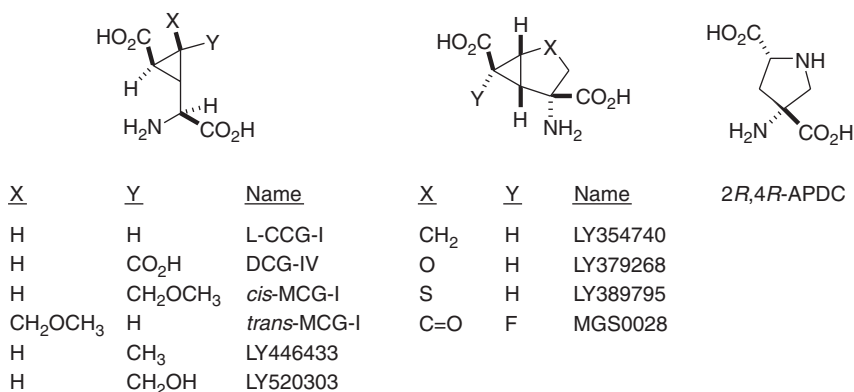


Figure 11.4 Group II selective orthosteric agonists.

($K_d = 160$ nM) [70]. In rat brain cortex homogenates, ^3H -DCG-IV bound to a single site with $K_d = 180$ nM and $B_{\text{max}} = 780$ fmol/mg protein, and this binding was potently displaced by the group II antagonist LY341495 and group II agonist LY354740 [71]. Thus, ^3H -DCG-IV appears to be a useful ligand for studies of mGlu2 (and likely mGlu3) in native tissues.

MCG-I: Placement of a methoxymethyl substituent on the cyclopropane ring of L-CCG-I (see Fig. 11.4) has led to additional compounds with reported group II mGlu receptor selectivity. These include *cis*- and *trans*-MCG-I (Fig. 11.4), which like other group II mGlu agonists potently suppress monosynaptic excitations in the newborn rat spinal cord and/or forskolin-stimulated cAMP formation in cultured rat cortical neurons, with the *trans*-MCG-I isomer being more potent [72–74].

LY446433: The compound C3'-C2'-carboxycyclopropyl glycine was designed as a ring-opened version of LY354740 (Fig. 11.4) [75]. LY446433 was similar in potency and selectivity to LY354740 in vitro (8 and 38 nM EC_{50} at mGlu2 and mGlu3, respectively). When administered orally to rats it was also substantially more potent than LY354740 in the fear-potentiated startle model of anxiety.

LY520303: The compound (2S,1'S,2'R,3'R)-2-(2'-carboxy-3'-hydroxymethylcyclopropyl) glycine (Fig. 11.4) [76] is also a potent (low-nanomolar) agonist for mGlu2 and mGlu3 receptors with no appreciable affinity at ionotropic receptors but less group II mGlu selectivity ($\text{EC}_{50} = 10$ nM and 147 nM at mGlu8 and mGlu6, respectively). The high affinity for mGlu2/3 and 8 makes it unique for studying the contribution of mGlu8 activity along with group II agonist activity. When administered systemically LY520303 was active in anxiety and psychosis models (like mGlu2/3 selective agents) but has not been widely explored in other models to test if addition of mGlu8 activity changes the pharmacological profile or even offers some therapeutic advantage over mGlu2/3 agonists alone. Consistent with its in vitro profile described above, ^3H -LY520303 was shown to bind selectively to cells expressing human mGlu2, 3, or 8 receptors as well as rat forebrain membranes [77].

LY354740: As described above, a number of selective agonists for group II mGlu receptors have now been described. The conformationally restricted bicycloamino acid compound LY354740 ((1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate) (Fig. 11.4) [78] was the first group II selective agonist which has been shown to have potent pharmacological effects in animals following systemic administration (for a detailed review on this compound see [8]. LY354740 potently activates mGlu2 ($\text{EC}_{50} = 5$ nM) and mGlu3 ($\text{EC}_{50} = 24$ nM) receptors in vitro but has no appreciable effects on other mGlu subtypes or ionotropic glutamate receptors at two to four orders of magnitude higher concentrations [26, 79]. In rat hippocampus, LY354740 suppresses forskolin-stimulated cyclic adenosine monophosphate (cAMP) formation with about the same potency as was observed in mGlu3-expressing cells ($\text{EC}_{50} = 22$ nM), but LY354740 has no effect on phosphoinositide hydrolysis per se [80]. Thus, LY354740 is a highly potent and selective mGlu2/3 agonist at both recombinant human and native rat receptors. In rat brain tissue, ^3H -LY354740 has been shown to bind with high affinity ($K_d = 8$ nM). Specific ^3H -LY354740 in the rat brain exhibited a pharmacology and distribution consistent with the selective labeling of group II mGlu receptors [81]. Consistent with the role of group II mGlu receptors in suppression of glutamate transmission in vitro, systemic LY354740 has been shown to prevent veratridine-evoked glutamate and aspartate release in free-moving rats undergoing microdialysis of the striatum [82]. Orally administered LY354740

potently suppressed fear-potentiated startle responding in rats, indicating it has anxiolytic activity, but unlike other anxiolytics (e.g., diazepam), LY354740 did not produced central nervous system (CNS) depression at any dose [83]. Acute LY354740 administration blocked withdrawal reactions to multiple agents including morphine [84], nicotine [85], and diazepam [86]. However, subchronic administration of LY354740 itself did not produce withdrawal. LY354740 was reported to suppress the in vivo actions of phencyclidine (PCP) in rats [87, 88]. These data suggest that LY354740 may be useful for the treatment of various psychiatric disorders. More recent data in knockout animals have linked the anxiolytic acity of LY354740 (in the elevated plus maze test) to activation of both mGlu2 and mGlu3 [89], while the antipsychotic actions (reversal of PCP behaviors) has been linked to mGlu2 receptors (the role of mGlu3 was not reported) [90]. Using cFos as an index of neuronal activation, Linden et al. [91] found that the anxiolytic actions of LY354740 on the elevated plus maze was associated with stress-induced suppression of *c-fos* induction in the hippocampus. Furthermore, LY354740 per se induces c-Fos within GABAergic brake cells of the lateral portion of the central amygdala, suggesting that the anxiolytic actions of this compound may also be linked to preventing the stress-induced disinhibition of limbic outputs [91, 92].

As has been reported in rats [87], LY354740 attenuated the disruptive effects of an NMDA receptor antagonist on working memory in humans [93]. Also, LY354740 has been reported to reduce anxiety in panic patients that was induced by carbon monoxide [94] and reduced anxiety as measured by the Hamilton-A anxiety scale (HAM-A) in patients with generalized anxiety disorder [95].

Studies have shown that LY354740 was neuroprotective in the gerbil global ischemia model [96] and traumatic neuronal injury in vitro and in vivo [97]. However, LY354740 was not active following permanent focal ischemia in the rat [50]. Thus, the role of group II mGlu receptor agonists as novel neuroprotectants is not yet clear, in particular as it relates to specific therapeutic applications. The use of additional systemically active mGlu2/3 agonists such as LY379268 has also been useful to clarify the potential therapeutic applications for mGlu2/3 receptor agonists (see below).

LY379268/LY389795: The conformationally constrained heterocyclic compounds with either oxygen (LY379268) or sulfur (LY389795) in the 2 position of the bicyclohexane ring have been described as highly potent and systemically active mGlu2/3 agonists (see Fig. 11.4) [98]. In functional assays of human mGlu2- or mGlu3-expressing cells, LY379268 and LY389795 were 3–8 times more potent than LY354740. In a model of limbic seizures in mice induced by 1*S*,3*R*-ACPD [29], as shown previously for LY354740 [78], both LY379268 and LY389795 were anticonvulsant in this test when given parenterally (i.p.) [98]. In another study [88], LY379268 was compared to LY354740 in the PCP model of psychosis in rats. When administered by the subcutaneous (s.c.) route, both compounds blocked motor activations induced by 5 mg/kg PCP; however, LY379268 was about 3 times more potent than LY354740. In monoamine-depleted animals LY379268, similar to the atypical antipsychotic clozapine but unlike haloperidol, reduced PCP behaviors in rats [99]. Carter et al. [100] showed that LY379268 injected into the cortex or thalamus decreased neuronal injury in the retrosplenial cortex induced by the NMDA receptor antagonist MK-801. Therefore, these heterobicyclic compounds, like LY354740, have been useful agents to further validate the therapeutic prospects of

mGlu2/3 agonists following systemic administration in the treatment of psychosis. Likewise, LY379268 has been useful to further study neuroprotection in vivo [101]. Scheifer et al. [102] studied LY379268 in a transgenic mouse model of Huntington's disease. LY379268 increased survival time in these animals. Jones et al. [103] studied both LY379268 and LY389795 for analgesic effects following acute and repeat dosing. Their data suggested that mGlu2/3 receptors may be involved in the mechanisms of hyperalgesia, but tolerance develops rapidly to these effects.

MGS0028: (1*R*,2*S*,5*S*,6*S*)-2-Amino-6-fluoro-4-oxobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (Fig. 11.4) is another nanomolar potent and selective mGlu2/3 receptor agonist that has been shown to have systemic activity in the PCP model of psychosis when given orally to rats [104]. Takamori et al. [105] showed that MGS0028 was also antipsychotic in the conditioned avoidance response assay in rats when given orally. In this study, LY354740 was not active by the oral route in this test. It would appear based on these data that there are differences in mGlu2/3 receptor agonists in these tests that remain unresolved.

*2*R*,4*R*-APDC*: 2*R*,4*R*-4-Aminopyrrolidine-2,4-dicarboxylate (2*R*,4*R*-APDC; LY-314593; see Fig. 11.4) is a conformationally constrained glutamate analog in which a pyrrolidine ring structure is present rather than the cyclopentane group in ACPD [106]. The presence of this nitrogen in the ring produced a group II selective compound with about the same potency as 1*S*,3*S*-ACPD (see [8]). In human mGlu2- or mGlu3-expressing cells 2*R*,4*R*-APDC suppresses forskolin-stimulated cAMP formation with EC₅₀ values of ~400 nM, but there were no appreciable effects on group I (mGlu1 or mGlu5) or group III (mGlu4, mGlu7, mGlu6, or mGlu8) receptors at up to 100 μM [26, 107, 108]. In rat brain slices, 2*R*,4*R*-APDC selectively suppresses forskolin-stimulated cAMP formation at concentrations (1–100 μM) having no effect on phosphoinositide hydrolysis per se [109]. 2*R*,4*R*-APDC, like the other group II selective agonist 1*S*,3*S*-ACPD, was shown to inhibit the development of kindled seizures in rats [110, 111], suggesting a role for group II mGlu receptors in epilepsy. Fisher and Coderre [32] reported that, unlike the group I agonist 3,5-DHPG, 2*R*,4*R*-APDC does not induce spontaneous nociceptive behaviors in rats. These in vivo studies are consistent with the highly selectivity of 2*R*,4*R*-APDC for group II mGlu receptors in vitro or when directly applied in vivo. The potency (micromolar) and selectivity of 2*R*,4*R*-APDC make it a useful in vitro tool. However, the discovery of more potent and systemically acting mGlu2/3 agonists essentially limits the use of this compound to in vitro studies or direct injection into the tissue.

11.2.4 Group II Selective Orthosteric Antagonists

MCCG: (2*S*,1'*S*,2'*S*)-2-Methyl-2-(2'-carboxycyclopropyl)glycine (MCCG; Fig. 11.5) is the α-methyl derivative of the potent group II mGlu receptor agonist L-CCG-I. Pharmacological studies have demonstrated that this simple substitution produces an antagonist for group II mGlu receptors with high micromolar affinity. In the neonatal spinal cord preparation, MCCG blocked the presynaptic depressant effects of 1*S*,3*S*-ACPD and L-CCG-I, while having minimal effects on the depressant effects of L-AP4. MCCG had no effect on the depolarizing response to 1*S*,3*R*-ACPD, indicating no appreciable group I mGlu receptor activity [112]. This group II selective antagonist profile has also been observed in recombinant systems expressing

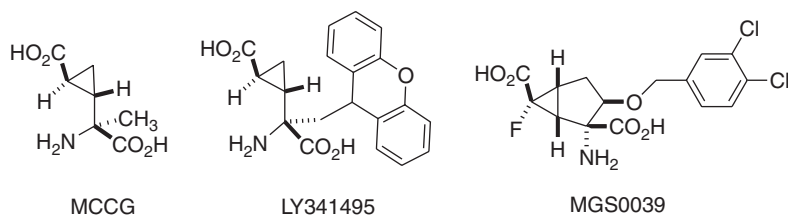


Figure 11.5 Group II selective orthosteric antagonists.

mGlu1, mGlu2, and mGlu4 cloned receptors [8, 113]. However, in adult rat cerebral cortex slices, MCCG acted as a group/partial agonist, as indicated by suppression of forskolin-stimulated cAMP formation to about 80% of that observed with maximally effective concentration of L-CCG-I [114]. The actions of MCCG on mGlu3 receptors would likely also contribute along with mGlu2 receptors in cAMP response in the rat brain.

LY341495: The α -xanth-9yl-methyl-carboxycyclopropylglycine (LY341495, Fig. 11.5), represented the most potent/optimal substituent from a series of phenylethyl- or diphenylethyl-substituted compounds [115, 116]. Full characterization of LY341495 across human mGlu receptor subtypes indicated that LY341495 is a competitive antagonist with low nanomolar potency at mGlu2 and mGlu3 receptors but at higher concentrations (high nanomolar to micromolar) LY341495 will antagonize mGlu receptor responses in cloned group I and group III receptors [8, 117]. LY341495 had no measurable affinity for ionotropic glutamate receptors. The high potency and selectivity for group II receptors when used at submicromolar concentrations but measurable antagonist actions at other receptors at higher (μ M) concentrations have made LY341495 a highly useful concentration-dependent agent for investigating the potential role of multiple mGlu receptor subtypes. For example, ³H-LY341495 selectively binds to recombinant human and native rat mGlu2 and mGlu3 receptors (K_D values of 1.7 and 0.75 nM, respectively), with a pharmacology (at 1 nM) indicating specific labeling of these receptors in either cells expressing cloned mGlu2/3 subtypes [118] or in the rat brain [119]. This makes ³H-LY341495 a highly useful ligand (under these conditions) for quantitating mGlu2/3 receptor expression, investigating the affinity of other compounds for mGlu2 or mGlu3 binding sites, and investigating the physiological/pathological regulation of these binding sites. Higher concentrations of LY341495 that block all mGlu receptor subtypes ($\sim 100 \mu$ M) have been used to rule out mGlu receptor involvement in physiological processes [120].

LY341495 has also been used *in vivo*, where relatively low parenteral doses (0.3–1 mg/kg) have been shown to block the pharmacological actions of selective group II mGlu receptor agonists. Examples include the elevated plus maze test for anxiety in mice where 0.3 mg/kg *i.p.* LY341495 reversed the anxiolytic actions of LY354740 while having no effect on its own [116] and the PCP model of psychosis in rats where LY341495 (1 mg/kg *s.c.*) alone had no effect on PCP-evoked motor activations, but the inhibitory actions of LY379268 against PCP were completely reversed [88]. These potent and selective actions of LY341495 make it a useful pharmacological agent to study group II mGlu receptor functions. In particular, systemic administration of LY341495 *per se* (0.6–3 mg/kg *i.p.*) has been used to probe how much endogenous

tone exists at mGlu2/3 receptors in vivo in mice. Linden et al. [89] used c-Fos as a marker of neuronal activity and found a widespread pattern of activation suggesting considerable endogenous tone at perisynaptic mGlu2/3 receptors, even under low-stress conditions where synaptic glutamate release/spillover would be expected to be minimal. Xi et al. [121] showed that systemically administered LY341495 increased extracellular glutamate levels, and this was reversed by APDC.

The ability to enhance neuronal activity by blockade of endogenous mGlu2/3 receptors is also supported by additional behavioral and biochemical data. LY341495 administration per se has been shown to be anxiogenic in the mouse elevated plus maze [122], increase locomotor activity in mice [123], stimulate waking and fast electroencephalographic (EEG) power in rats [124], exacerbate behavioral signs and locus ceruleus neuronal firing associated with morphine withdrawal in rats [125], and prevent the ability of *N*-acetylcysteine to inhibit the reinstatement of cocaine seeking in rats [126]. Studies with LY341495 and MGS0039 also suggest that mGlu2/3 receptor antagonists have novel antidepressant-like potential (see below).

MGS0039: (1*R*,2*R*,3*R*,5*R*,6*R*)-2-Amino-3-(3,4-dichlorobenzyloxy)-6-fluorobicyclo [3.1.0] hexane-2,6-dicarboxylic acid (Fig. 11.5), like LY341495, has low nanomolar potency at both mGlu2 and mGlu3 (2.4 and 4.5 nM, respectively) [127]. MGS0039 has very low relative affinities for other mGlu receptors and has been shown to be active when administered systemically to mice and rats. Chaki et al. [128] showed MGS0039 to have antidepressant activity in the rat forced-swim test and mouse suspension test in mice. Subsequently, Shimazaki et al. [129] reported MGS0039 was anxiolytic in the marble-burying test, unlike the mGlu2/3 receptor antagonist LY341495 (the reasons for this are not clear). In support of the hypothesis that mGlu2/3 antagonists are antidepressant, Yoshimizu et al. [130] reported that chronically administered MGS0039 increased cell proliferation (BrdU labeling) in the adult mouse hippocampus. The work of Karasawa et al. [131] suggests that the antidepressant actions of MGS0039 involves activation of AMPA receptors, which drives increases in serotonin neurotransmission. In their study, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline (NBQX) blocked the antidepressant effects of MGS0039 in the tail suspension test and MGS0039 induced increases in serotonin release in the rat prefrontal cortex. Increases in serotonin neurotransmission subsequent to mGlu2/3 receptor antagonism has also been reported for MGS0039 and LY341495 using electrophysiology [132, 133].

11.2.5 Group III Selective Orthosteric Agonists

L-AP4/L-SOP: Simple bioisosteric replacement of the distal carboxylic acid moiety on glutamic acid has led to novel and interesting group III mGlu selective agonists. The most well studied and notable of these compounds are L-4-phosphono-2-aminobutyric acid (L-AP4) and L-serine-*O*-phosphate (L-SOP) (see Fig. 11.6). Prior to the recognition that mGlu receptors existed, L-AP4 was reported to suppress excitatory transmission in multiple excitatory synapses, including the lateral perforant path [134], mossy fiber pathway [135, 136], lateral olfactory tract [137], and retina [138]. Ganong and Cotman [139] showed that this presynaptic inhibitory action of L-AP4 in the lateral perforant path was also produced by L-SOP. This so-called L-AP4 receptor remained somewhat an enigma until the cloning of mGlu receptor subtypes, when it was recognized that these compounds act as relatively potent and

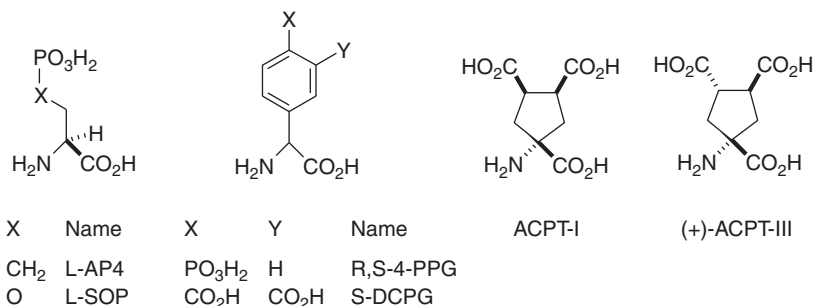


Figure 11.6 Group III selective orthosteric agonists.

somewhat selective agonists for mGlu4, mGlu6, mGlu7, and mGlu8 receptors (see [140]). As each of the L-AP4-sensitive members of the cloned group III mGlu receptors (e.g., mGlu4, mGlu7, and mGlu8) are found to exist presynaptically in L-AP4-sensitive excitatory pathways, it is possible that in a given synapse one or more of the group III receptors function as negative-feedback receptors that regulate glutamate release. Studies have attempted to understand which L-AP4-sensitive subtype(s) are responsible for suppression of excitatory transmission in specific preparations (see [140, 141]). For example, the L-AP4 receptor in the retina has been suggested to be mGlu6, as this receptor shows selective expression in this tissue [142]. L-AP4 and L-SOP are each nanomolar to low micromolar potent agonists for multiple group III mGlu receptor subtypes, including mGlu4, mGlu6, and mGlu8 (see [8, 140]). As L-AP4 is relatively inactive at cloned group I or group II mGlu receptors, this has made this compound “diagnostic” for the involvement of group III receptors in cellular functions. However, millimolar concentrations of L-AP4 are required to activate mGlu7 receptors, and these concentrations have also been shown to activate cloned mGlu2 and mGlu3 receptors (see [8]). Furthermore, the selectivity of L-SOP across all cloned mGlu receptors needs to be better established. Clearly, better and more subtype selective agonists for group III mGlu receptors than L-AP4 or L-SOP are desirable to study the role of specific group III mGlu receptors in the regulation of excitatory synaptic transmission. In this regard, additional agents targeted at advancing group III mGlu receptor pharmacology have recently been described and are discussed below.

(R,S)-PPG: The phosphono-substituted phenylglycine compound *(R,S)*-4-phosphonophenylglycine [(*R,S*)-PPG], Fig. 11.6] has been shown to be a potent and selective mGlu receptor agonist at each cloned group III receptor subtype with the following order of potency: mGlu8 ($EC_{50} = 0.2 \mu M$) > mGlu6 = mGlu4 ($EC_{50} = 5 \mu M$) > mGlu7 ($EC_{50} = 185 \mu M$). Importantly, (*R,S*)-PPG is relatively inactive at cloned group I and group II mGlu receptors or ionotropic glutamate receptors [143, 144]. Separation of the enantiomers demonstrated that the mGlu8 agonist activity resides exclusively in the (+)-isomer [144]. Similar to other phosphono-amino acids, (*R,S*)-PPG showed micromolar potency at Ca^{2+}/Cl^{-} dependent binding sites in the rat brain. Consistent with other group III agonists such as L-AP4, (*R,S*)-PPG was neuroprotective against N-methyl-D-aspartate (NMDA)-mediated excitotoxicity in cultured neurons in vitro, and when coinjected with NMDA into the rat striatum in vivo. While not efficacious in protecting hippocampal neurons in global or focal ischemia models, [(*R,S*)-PPG]

300 nmol, i.c.v.] decreased quinolinic acid-induced striatal lesion size in the rat [145]. (*R,S*)-PPG was also shown to be fully protective against maximal electroshock seizures in mice when administered i.c.v. (173 nmol) but did not protect when given parenterally (100 mg/kg i.p., or 10 mg/kg i.v.) in the same test. In subsequent studies, (*R,S*)-PPG was anticonvulsant against sound-induced seizures in the DBA/2 mouse [146]. These studies with (*R,S*)-PPG further support a role for group III agonists as novel agents to treat seizure or neurodegenerative disorders (for a review on this subject (see [147]).

DCPG: (*S*)-3,4-Dicarboxyphenyl glycine (Fig. 11.6) represents the first subtype selective mGlu8 receptor agonist with an $EC_{50} = 31$ nM for activation of recombinantly expressed human mGlu8a receptors but no activity at other human mGlu receptor subtypes (1–7) at up to 1000 nM [148]. DCPG appears to be a useful compound to study the pharmacology associated with selective mGlu8 receptors both in vitro and in vivo. For example, DCPG was shown to depress the fast component of the dorsal root-evoked ventral root potential in the neonatal rat spinal cord preparation [148, 149]. Systemic administration of (*S*)-DCPG 30 or 100 mg/kg i.p. in the mouse increased c-Fos expression in certain stress-associated brain regions (central amygdala, paraventricular nucleus of the hypothalamus), and these changes were absent in the mGlu8 receptor knockouts [150]. Moldrich et al. [151] showed that *S*-DCPG was anticonvulsant in the DBA/2 mouse (i.c.v. injection) but that the racemic compound (*R,S*) was more potent, likely due to the AMPA receptor antagonist activity of the *R* isomer. Interestingly, (*R,S*)-DCPG 60–100 mg/kg i.p. had a typical (vs. atypical) antipsychotic profile in mice, but it is not clear if this involves its mGlu8 agonist and/or AMPA receptor activities [152].

ACPTs: Acher et al. [153] have reported on the four stereoisomers of 1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT). Two of these isomers, ACPT-I and ACPT-III (Fig. 11.6), were micromolar potent agonists for mGlu4a, with weak antagonist activities at mGlu1 and mGlu2. ACPT-I demonstrated anticonvulsant activity in multiple seizure models when given by an i.c.v. route [154]. These compounds may be useful to explore group III mGlu receptors; however, their activity across other glutamate receptor subtypes has not been fully characterized.

11.2.6 Group III Selective Orthosteric Antagonists

In general, group III mGlu receptor antagonists represent the least developed area of mGlu receptor pharmacology. Many agents with group III antagonist activity also possess group II mGlu receptor antagonist activity and/or their profile across all four group III receptors has not been described. Nevertheless, the actions of these agents which block group III mGlu subtypes can be compared to the effects of more selective group II agonists to get an indication of the possible involvement of group III mGlu receptors in native tissues. Furthermore, the ability of a compound to reverse the actions of selective group III agonists such as L-AP4 in native tissue is useful information suggesting group III involvement. With these caveats, certain compounds targeted at these receptors have emerged as useful agents and are discussed below. For a more detailed discussion of the structure–activity relationship of other compounds related to the ones described below, see Schoepp et al. [8].

MAP4/MSOP: (*S*)- α -Methyl-2-amino-4-phosphonobutanoic acid (MAP4) and (*R,S*)- α -methylserine-*O*-phosphate (MSOP) are the α -methyl analogs of the group III selective agonists L-AP4 and L-SOP, respectively (see Fig. 11.7). In the rat

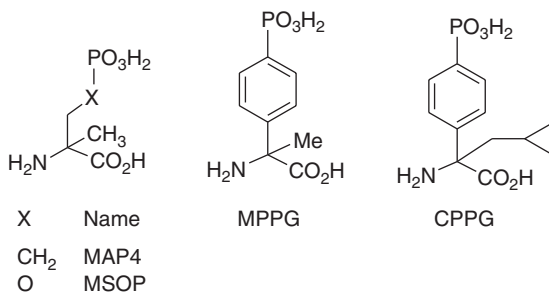


Figure 11.7 Group III selective orthosteric antagonists.

neonatal spinal cord preparation where these compounds were initially described, both were moderately potent group III selective competitive antagonists, in that they selectively reversed the depressant effects of L-AP4 [112, 155–157]. Likewise, in the rat thalamus *in vivo* [158], CA1 region of the hippocampus [159], and hippocampal lateral perforant path [160], MAP4 selectively blocked the actions of L-AP4. Thus, at least in these preparations, there appears to be a group III L-AP4-activated mGlu receptor subtype that is sensitive to MAP4 and/or MSOP. Studies indicate that MAP4 and MSOP are antagonists at mGlu4 receptors. However, the profile of MAP4 and MSOP across all eight receptors is not known, and there are some discrepancies in their activities across different laboratories (see [8]). For example, data in certain preparations suggest that MAP4 may have group II and/or group III mGlu receptor agonist/partial agonist activities. In adult rat cortical slices MAP4 potentially suppressed forskolin-stimulated cAMP formation ($EC_{50} = 0.11 \mu\text{M}$) [114], and in contrast to others [141], Knopfel et al. [113] found MAP4 to be a partial agonist at rat mGlu4a receptors. Thus, the antagonist actions of these agents should not be considered indicative of the involvement of a group III mGlu receptor in physiological or pathological processes involving glutamate.

MPPG: (*R,S*)- α -Methyl-4-phosphonophenylglycine (Fig. 11.7) is the phospho-substituted version of MCPG. As the substitution of the distal carboxy group in glutamate with a phosphono acidic bioisostere (producing L-AP4) leads to group III activity, one could suggest that a similar change to MCPG might produce group III antagonist activity. Indeed, MPPG has been shown to be a group III mGlu receptor antagonist in a number of test systems, as indicated by selective reversal (generally at 50–200 μM concentrations) of the actions of L-AP4 in rat spinal cord [20], the ventrobasal thalamus [161], lateral perforant path of the hippocampus [162], and L-AP4 suppression of forskolin-stimulated cAMP formation in adult rat cortex [163]. However, MPPG appears to act on group II receptors, as at similar or higher concentrations it has been shown to also reverse the actions 1*S*,3*S*-ACPD in the spinal cord [155] and L-CCG-I suppression of forskolin-stimulated cAMP formation in the rat cortex [163]. Studies in recombinant mGlu receptors have shown that MPPG is both a group II and group III mGlu receptor antagonist. It is a moderately potent antagonist at rat mGlu2 and mGlu4 receptors [156, 164–166]. Wu et al. [26] compared its actions across human group III receptors in the same cell line and found that MPPG most potently blocked mGlu8 receptors ($IC_{50} = 47 \mu\text{M}$) followed by mGlu7 ($IC_{50} = 528 \mu\text{M}$) and mGlu4 ($IC_{50} = 540 \mu\text{M}$). Thus, in general, the

usefulness of MPPG is limited by lack of good selectivity and potency for the group III mGlu receptors.

CPPG: (*R,S*)- α -Cyclopropyl-4-phosphonophenylglycine (CPPG) differs from MPPG by the incorporation of a cyclopropyl group at the α carbon rather than a methyl (Fig. 11.7). With this change, the potency of CPPG as an antagonist for both group II and group III mGlu receptors was greatly increased in the rat cerebral cortex. CPPG antagonized L-AP4 and L-CCG-I inhibitions of forskolin-stimulated cAMP formation with IC_{50} values of 2.2 and 46 nM, respectively. However, it only weakly ($K_B = 650 \mu M$) blocked group I agonist stimulations of phosphoinositide hydrolysis [167]. In membranes prepared from rat mGlu4a-expressing cells, CPPG displaced 3H -AP4 binding ($K_i = 23 \mu M$) with about three fold greater potency than MPPG or MAP4 [168]. However, the micromolar potency at mGlu4a receptors is at odds with nanomolar antagonist potency observed against L-AP4 second messengers in rat brain.

11.3 SUBTYPE SELECTIVE ALLOSTERIC POTENTIATORS AND ANTAGONISTS

The availability of recombinant mGlu subtypes, effective cellular expression strategies, and advances in functional assay formats for G-protein-coupled receptors have allowed for the high-throughput screening of compound libraries against individual mGlu receptor subtypes. This approach has resulted in the identification of a number of structurally novel non-amino acid allosteric modulators of the mGlu receptors. In most cases the positive allosteric modulators and allosteric antagonists act within the heptahelical (7-TM) domain of these receptors, rather than the glutamate recognition site within the amino-terminal segment. Thus, the antagonists are noncompetitive against glutamate and the positive allosteric modulators do not displace orthosteric ligands.

The majority of positive allosteric modulators characterized have acted as potentiators of glutamate in both clonal cell lines and native tissue assays, rather than simply as allosteric agonists. Rather, the potentiators typically act to enhance the binding affinity of orthosteric agonists, increasing the sensitivity of a mGlu-mediated effect in clonal cells and native tissues. Interestingly, many of the potentiators and allosteric antagonists alike demonstrate a high degree of mGlu subtype selectivity even against highly related mGlu receptors within the same group. Thus they may serve as highly useful pharmacological tools for delineating mGlu subtype-specific function(s). In addition, compounds with mixed actions (potentiator vs. antagonist) and unique selectivity profiles against mGlu subtypes across groups I, II, and III have been discovered. Examples of these very novel and important pharmacological tools for select receptors in groups I, II, and III are provided below.

11.3.1 mGlu1 Allosteric Potentiators

The first mGlu receptor potentiators described were for the mGlu1 subtype, for example, RO 67-7476 and RO 67-4853 (Fig. 11.8; (9*H*-xanthene-9-carbonyl)-carbamate butyl ester and (*S*)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine,

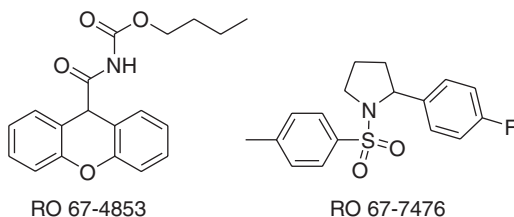


Figure 11.8 mGlu1 allosteric potentiators.

respectively] [169]. Both act to enhance a submaximal effect of glutamate or the orthosteric agonist DHPG in the rat mGlu1a receptor expressing cells with potentiation EC_{50} values of 174 and 69 nM for RO 67-7476 and RO 67-4853, respectively. Each is selective for the rat mGlu1 receptor versus mGlu5, mGlu2, mGlu4, and mGlu8 receptors, but interestingly, RO 67-7476 did not show potentiation of the recombinant human mGlu1a receptor, while RO 67-4853 was equipotent and efficacious at both the rat and human receptors. Systematic testing in chimeric and point mutants of the group I receptors localized the species selectivity to a single amino-acid change within transmembrane region (TM) 5 where a valine in the rat mGlu1a receptor is a leucine in both the human mGlu1 and rat mGlu5 receptors. The group went on to show that this change (valine to leucine in TM5) in combination with two changes in TM3 (a serine to phenylalanine and cystine to serine) accounts for the subtype selectivity for rat mGlu1 versus mGlu5 as well. Though activity for both RO 67-7476 and RO 67-4853 was confirmed in native tissue preparations from rat cerebellum or hippocampus, the utility of these novel pharmacological tools for in vivo behavioral and biochemical applications remains unknown. However, a recent patent application disclosure from Hoffman-LaRoche describes a series of related oxazol-2-yl-amide xanthenes with less than 200 nM potency as mGlu1 potentiators and decreased metabolism in liver microsomes [170, 171]. These new allosteric modulators may well represent useful tools in studying the effects of mGlu1 potentiators.

11.3.2 mGlu1 Allosteric Antagonists

CPCCOEt: 7-Hydroxyiminocyclopropan[b]chromene-1 α -carboxylic acid (Fig. 11.9) exhibits micromolar potency ($IC_{50} \sim 23 \mu M$) as a noncompetitive inhibitor of functional responses at human mGlu1 receptors but did not exhibit any appreciable activities at mGlu2, mGlu4a, mGlu5a, mGlu7b, or mGlu8a receptors when tested at up to 100 μM [172–175]. CPCCOEt does not directly interact at the glutamate binding domain, as it did not displace 3H -glutamate binding to the receptor [175]. Interestingly, it appears to interact at a region downstream from the N-terminal extracellular domain, specifically involving residues Thr815 and Ala818 within TM-VII of mGlu1, as introduction of these residues into the mGlu5a receptor introduced sensitivity for CPCCOEt inhibition to this receptor. Likewise, substitution of the corresponding mGlu5a residues for the critical residues on mGlu1 resulted in loss of CPCCOEt inhibition [175]. These studies with CPCCOEt illustrate a highly specific molecular interaction of this agent at a site distal to the glutamate binding region.

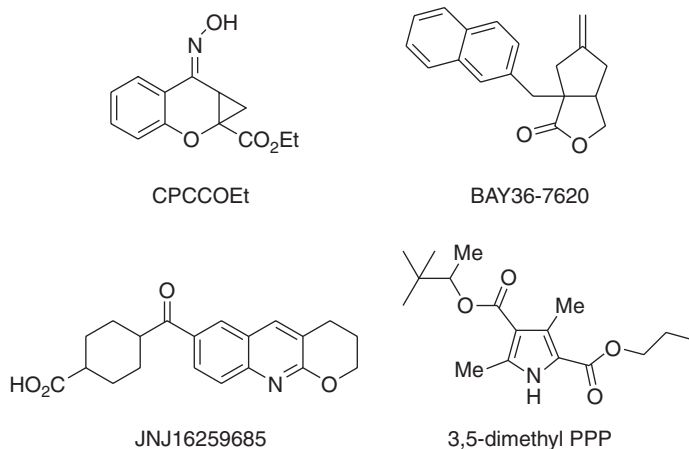


Figure 11.9 mGlu1 allosteric antagonists.

Since the recognition of CPCCOEt as an allosteric antagonist, several highly potent and structurally distinct mGlu1 negative modulators have been described. For instance, Fig. 11.9 illustrates three such mGlu1 antagonists with nanomolar potency in functional assays (calcium mobilization) using mGlu1-expressing cells: 160 nM BAY36-7620 [(3a*S*,6a*S*)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[*c*]furan-1-on] [176]; 3 nM JNJ16259685 [(3,4-dihydro-2*H*-pyrano [2, 3]*b* quinolin-7-yl) (*cis*-4-methoxycyclohexyl) methanone] [177]; and 16 nM IC₅₀ 3,5-DiMePPP [3,5-dimethyl pyrrole-2,4-dicarboxylic acid 2-propyl ester 4-(1,2,2-trimethyl-propyl) ester] [178]. All three were characterized and found to be non-competitive antagonists, with at least 100-fold selectivity for mGlu1 receptor versus the other mGlu receptors examined. BAY36-7620 did not displace the mGlu1 orthosteric agonist [³H]-quisqualate and the binding domain for BAY36-7620 and 3,5-DiMePPP was localized to the transmembrane region by testing with chimeric receptors. Furthermore, JNJ16259685 was found to displace with high affinity a previously characterized radiolabeled allosteric antagonist [³H]-R214127 (1-(3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-2-phenyl-1-ethanone) [177].

In vivo, BAY36-7620 was efficacious in an acute subdural hematoma rat model (0.01 mg/kg/h i.v. infusion) as well as protective against pentylenetetrazole-induced convulsions in mice (10 mg/kg i.v.) [179]. 3,5-DiMePPP was efficacious in multiple pain models at 0.3–10 mg/kg i.p., including a mouse formalin pain model, a rat carrageenan pain model, and a rat chronic nerve injury pain model [178]. Similarly, utilizing an ex vivo autoradiography method, JNJ16259685 (0.04 mg/kg s.c.) displaced cerebellar binding of the allosteric radiolabeled antagonist [³H]-R214127 [177]. Given these results, the three compounds promise to be very useful pharmacological tools in determine the importance of mGlu1 antagonism in multiple disease states.

11.3.3 mGlu2 Allosteric Potentiators

The first positive allosteric modulators for the mGlu2 receptor are typified by the (3-pyridinyl)methylsulfonamide 4-MPPTS or LY487379 shown in Fig. 11.10

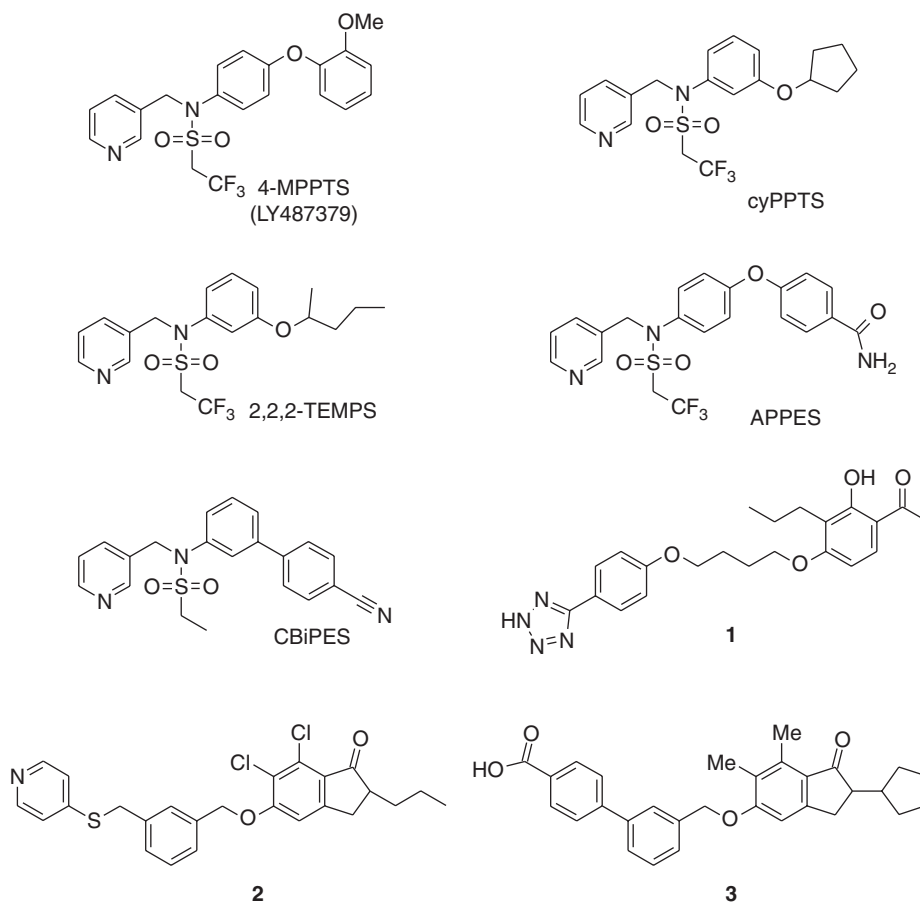


Figure 11.10 mGlu2 allosteric potentiators.

(2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide) [180, 181]. As with the mGlu1 potentiators, 4-MPPTS and its close analog 3-MPPTS (2,2,2-trifluoro-*N*-[3-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide) act to increase the potency of orthosteric agonists as indicated by glutamate curves shifting leftward in functional assays and increased affinity of mGlu2 agonists such as [³H]-DCG-IV [2*S*,1'*R*, 2'*R*, 3'*R*-2-(2', 3'-dicarboxycyclopropyl) glycine] [181, 182]. Here, as well, testing in chimeric and point mutation altered mGlu2 receptors has indicated that the binding domain of the potentiators is within the transmembrane domain with a critical role for an asparagine in TM5 [181].

Structure-activity relationships (SAR) within this series of compounds has led to some very potent potentiators, such as cyPPTS (24 nM; 2,2,2-trifluoro-*N*-[3-(cyclopentyloxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide) [183]. Indeed, cyPPTS typifies one important aspect of potentiators versus orthosteric agonists, namely an ability to function in a state-dependent manner. In rat brain slices, corticostriatal excitatory postsynaptic potentials (EPSPs) could be inhibited with the mGlu2/3 receptor orthosteric agonist LY354740 irrespective of frequency of axonal stimulation.

However, EPSPs are inhibited with the potentiator cyPPTS only when the higher frequency stimulus was given (conditions of more glutamate release) [182]. Thus, potentiators might be expected to selectively impact neuronal pathways only under conditions of excessive glutamate release (i.e., potentially disease states) while not affecting resting states (i.e., fewer side effects).

In animal models, mGlu2 potentiators such as 4-MPPTS, 2,2,2-TEMPS (2,2,2-trifluoro-*N*-[3-(1-methyl-butoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide), 4- APPEs (*N*-[4-(4-carboxyamidophenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide), and CBiPES (*N*-[4'-cyano-biphenyl-3-yl]-*N*-(3-pyridinylmethyl)ethanesulfonamide) have all shown efficacy in one or more models of anxiety and/or psychosis. For instance, when administered subcutaneously both 4-MPPTS and APPEs were anxiolytic-like in a rat potentiated startle paradigm, as was CBiPES in reducing stress-induced hyperthermia in mice, both animal models of anxiety disorders [180, 182]. CBiPES was also efficacious in a PCP-induced locomotor activity model commonly used to test for antipsychotics [182], and pretreatment with 2,2,2-TEMPS was effective in limiting the norepinephrine dialysate released by ketamine treatment in the hippocampus of rats [184].

More recently a second chemical series of mGlu2 potentiators has been described. This second series is typified by the phenyl-tetrazolyl acetophenone 1-(2-hydroxy-4-{4-[4-(2*H*-tetrazol-5-yl)-phenoxy]-butoxy}-3-propyl-phenyl)ethanone (compound **1**, Fig. 11.10) [185, 186]. Compound **1** is reported to be a selective mGlu2 potentiator with an EC₅₀ of 300 nM and no allosteric or orthosteric activity against mGlu3, 1, 4, 5, 7, or 8. However, when given to rats at 20 mg/kg i.p. compound **1** showed very low brain-to-plasma ratios. It should also be cautioned that some phenyl-tetrazole acetophenones such as compound **1** are reported to be antagonists at the leukotriene receptor cystinyl leukotriene 1 (CysLT1) subtype normally activated by leukotriene D4 (LTD4) and may have some activity at one or more peroxisomal proliferation activating receptors (PPARs). Recently Pinkerton and co-workers [187–189] reported on related molecules with similar potencies to **1**, including (±)-6,7-dichloro-2,3-dihydro-2-propyl-5-[(3-[(4-pyridinylthio)methyl]phenyl)methoxy]-1*H*-Inden-1-one (compound **2**, Fig. 11.10) and (±)-5-[(4'-carboxy-[1,1'-biphenyl]-3-yl)methoxy]-2,3-dihydro-6,7-dimethyl-2-pentyl-1*H*-Inden-1-one (compound **3**, Fig. 11.10). In contrast to compound **1**, the brain penetration for compounds **2** and **3** were greatly improved at 2 h with a 20 mg/kg i.p. dose. When tested in a ketamine-induced locomotor activity model of psychosis a 40-mg/kg i.p. dose of compound **2** partially reversed the ketamine response [188], while a 20 mg/kg i.p. dose of compound **3** was fully efficacious. More experiments are needed, but these initial results suggest that CBiPES and compound **3** may be useful pharmacological tool to explore the utility of mGlu2 potentiators.

11.3.4 mGlu2/3 Allosteric Antagonists

To date only one chemical series with negative allosteric activity (i.e., noncompetitive antagonism) has been identified for the group II mGlu receptors. Based on the available patent applications and a Society for Neuroscience abstract, RO 71-8218 (Fig. 11.11; 8-(4-fluoro-phenylethynyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[*b*][1,4]diazepin-2-one) [190, 191] shows nanomolar potency as a non-competitive antagonist against mGlu2 and mGlu3 receptors and was selective versus

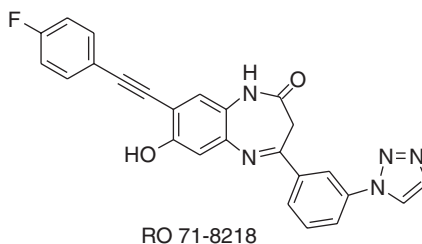


Figure 11.11 mGlu2 allosteric antagonists.

mGlu1, 4, 5 and 8 as well as ionotropic glutamate receptors. Interestingly RO 71-8218 also partially displaced the binding of an mGlu2/3 orthosteric agonist [^3H]-LY354740, suggesting a possible modulation of the glutamate site by binding to a distal allosteric site. Alternatively, RO 71-8218 could share a partially overlapping binding domain with the glutamate site in the amino-terminal segment. Here, again, too little information is available to definitively determine the mechanism of antagonism (allosteric vs. orthosteric) or the utility of these antagonists as pharmacological tools in vitro or in vivo.

11.3.5 mGlu4 Allosteric Potentiators

Progress in the identification and characterization of allosteric modulators for the group III mGlu receptors (4, 6, 7, and 8) has been somewhat slower in coming, although an antagonist of the mGlu7 receptor (see below) and a handful of mGlu4 potentiators have been described. Interestingly two of the mGlu4 potentiators were first characterized as mGlu5 receptor allosteric antagonists (vide infra), namely 2-methyl-6-(2-phenylethenyl)pyridine (SIB-1893) and MPEP (see Fig. 11.14 below [192]). However, subsequent testing found that both are also mGlu4 potentiators though much less potent compared to their nanomolar potency as allosteric antagonists at the mGlu5 receptor. Nevertheless, both SIB-1893 and MPEP increase the potency and/or maximal effect of the orthosteric agonists, such as L-AP4 and L-glutamate, in a clonal cell line expressing human mGlu4 receptors. As anticipated with an allosteric mechanism neither SIB-1893 nor MPEP displaced the orthosteric agonist [^3H]-L-AP4. Rather, 50–100 μM SIB-1893 significantly increased the affinity of [^3H]-L-AP4, similar to what has been reported for the mGlu2 potentiators.

Interestingly, another structural class of mGlu4 potentiators is also structurally related to an allosteric group I antagonist. Specifically CPCCOEt is an allosteric mGlu1 antagonist (as described above; Fig. 11.9) while a closely related structure, PHCCC (*N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1 α -carboxamide; Fig. 11.12) is an

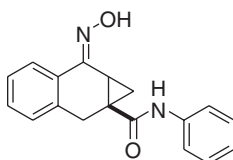


Figure 11.12 mGlu4 allosteric potentiator.

allosteric potentiator of mGlu4 [193, 194]. PHCCC is not, however, selective for the mGlu4 receptor, showing similar micromolar potency for mGlu1 antagonism and less than 10-fold selectivity for antagonism of mGlu2, mGlu8, and mGlu5. In native tissue systems, PHCCC was neuroprotective in primary mixed neuronal/glial cortical cultures against NMDA- or β -amyloid protein (25–35)-induced toxicity [193] and potentiated the inhibitory response of L-AP4 in striatal pallidal synapses from rat brain slice preparations [194]. Given the latter effect, Marino and co-workers [194] went on to demonstrate that direct intracerebroventricular injection of PHCCC, but not CPCCOEt, dramatically reversed the akinesia induced by acute dopamine depletion with reserpine, a model of Parkinsonism-related motor deficits. Similarly, direct injection of PHCCC into the basolateral amygdala of rats was effective in an anxiety model, the Vogel conflict drinking paradigm [195], though a partial response was also seen with the mGlu1 antagonists CPCCOEt, and a small but statistically insignificant decrease in shock threshold with both compounds makes interpretation of the results somewhat difficult. Taken together, the available results with PHCCC do suggest potentiators of mGlu4 hold therapeutic promise. However, more potent, selective, and systemically active mGlu4 potentiators will need to be discovered and characterized before any definitive conclusions can be made.

11.3.6 mGlu5 Allosteric Potentiators

3,3'-Difluorobenzaldazine (DFB; Fig. 11.13) was the first potent and selective positive allosteric modulator of mGlu5 to be described [196]. At concentrations up to 100 μ M, DFB is devoid of agonist activity at recombinant human mGlu5 receptors. However, in the presence of submaximal (300 nM; approximate EC_{10} concentration) glutamate, DFB produced a concentration-dependent activation of this receptor with an apparent EC_{50} value of 2.6 μ M and a maximal 3.1-fold potentiation of this submaximal glutamate challenge. Furthermore, DFB induces a 2-fold leftward shift of the orthosteric agonist (glutamate, 3,5-dihydroxyphenylglycine or quisqualate) concentration–response curves at recombinant human, recombinant rat, and native tissue (hippocampal) rat mGlu5 receptors *in vitro*. As expected, DFB did not influence the binding of the orthosteric site radioligand [3 H]-quisqualate but was partially effective (51% maximal efficacy) in displacing [3 H]-methoxy-PEPy (2-phenylethynylpyridine) binding from its TM7 binding site [197]. At 100 μ M, DFB displays mGlu4 and mGlu8 antagonist activity. Interestingly, minor structural modification of DFB led to both a noncompetitive mGlu5 antagonist and a ligand that displays neutral cooperativity with orthosteric agonists [196].

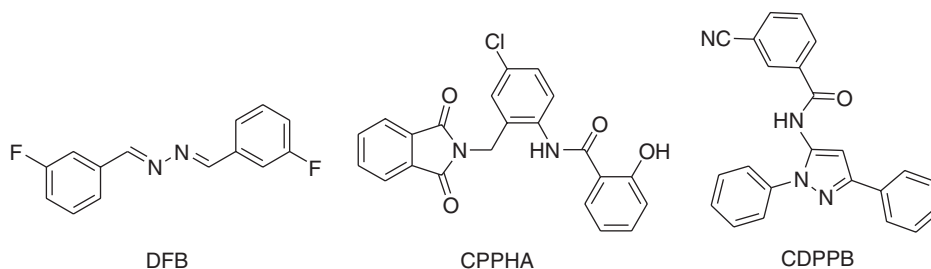


Figure 11.13 mGlu5 allosteric potentiators.

Another structurally distinct mGlu5 potentiator, CPPHA (*N*-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoinol-2-yl)methyl]phenyl)-2-hydroxybenzamide; Fig. 11.13) [198], was identified as a mGlu5 potentiator (human mGlu5 EC_{50} = 393 nM; 7-fold leftward shift in the glutamate concentration–response curve. CPPHA increased NMDA currents in hippocampal CA1 pyramidal cells in the presence of submaximal DHPG and increased depolarization of subthalamic neurons, again in the presence of submaximal DHPG. Interestingly, CPPHA does not influence the binding of either [3 H]-quisqualate or [3 H]-methoxy-PEPy, suggesting a unique site of action. Like DFB, CPPHA also demonstrates antagonist activity at both mGlu4 (IC_{50} = 12.7 μ M) and mGlu8 (IC_{50} = 7.5 μ M). The utility of CPPHA as a tool to study mGlu5 function may be limited owing to significant off-target cross reactivity with a number of non-glutamate targets at submicromolar concentrations, including PDE₆ (phosphodiesterase), δ - and κ -opiate receptors, and hERG (Human Ether-a-go-go Related Gene) potassium channels [198].

Most recently, CDPPB [3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; Fig. 11.13] has been reported to potentiate mGlu5 receptors with high potency (human mGlu5 EC_{50} = 10–27 nM) [199, 200] and a 9-fold leftward shift of the glutamate concentration–response curve [200]. CDPPB did not influence the binding of the orthosteric site radioligand [3 H]-quisqualate but was fully effective in displacing [3 H]-methoxy-PEPy binding from its TM7 binding site (K_i \sim 2 μ M). CDPPB produces an apparent mGlu5 agonist effect at concentrations significantly above those required for potentiator activity (agonist EC_{50} \sim 10 μ M, E_{max} \sim 35–35% of maximal glutamate response). The agonist effect of CDPPB was not blocked by the orthosteric antagonist LY341495 (see Fig. 11.5) but was fully inhibited by the allosteric antagonist MPEP (see Fig. 11.14). While CDPPB possesses weak antagonist activity versus mGlu8 (IC_{50} \sim 10 μ M) [199], it appears to be significantly more selective than CPPHA, possessing greater than 1 μ M potency (IC_{50} or K_i) against a broad panel of receptors, transporters, ion channels, and enzymes [200]. CDPPB demonstrates poor (\sim 3%) oral bioavailability in rats; however, following subcutaneous administration at doses of 10–60 mg/kg, brain levels of CDPPB (110–270 nM) are in the range of those that are fully efficacious in potentiating submaximal

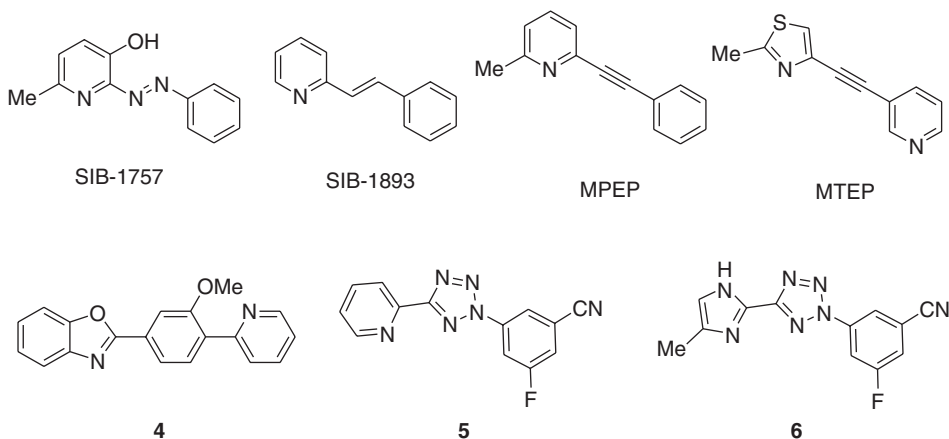


Figure 11.14 mGlu5 allosteric antagonists.

glutamate responses in vitro. In behavioral studies, CDPPB (10 mg/kg s.c.) had no effect on spontaneous locomotor activity in rats but was effective in decreasing amphetamine-induced ambulations [200]. Furthermore, CDPPB (10 and 30 mg/kg s.c.) had no effect on baseline acoustic startle responding but reversed amphetamine-induced deficits in prepulse inhibition (PPI) of startle [199, 200]. These results are consistent with the hypothesis that potentiation of mGlu5 receptors represents a novel approach for treatment of schizophrenia.

11.3.7 mGlu5 Allosteric Antagonists

SIB-1757 [6-methyl-2-(phenylazo)-3-pyridinol] and SIB-1893 [(*E*)-2-methyl-6-(2-phenylethenyl)pyridine] (Fig. 11.14) were the first noncompetitive antagonists of mGlu5 receptors to be described [201]. These were discovered by high-throughput screening of compounds for their ability to inhibit glutamate-induced intracellular calcium mobilization in a cell line expressing human mGlu5a receptors. SIB-1757 and SIB-1893 inhibit glutamate responses at human mGlu5a receptors with IC_{50} values of 0.37 and 0.29 μ M, respectively. Notably, neither analog elicited effects on human isoforms of mGlu1, mGlu2, mGlu3, mGlu4, mGlu6, mGlu7, or mGlu8 receptors at concentrations up to 100 μ M, nor were they active at recombinant AMPA, kainate, or NMDA receptors when tested up to 30 μ M. Consistent with their mGlu5 selectivity profile at human recombinant receptors, both SIB-1757 and SIB-1893 potently antagonized 3,5-DHPG-induced phosphoinositide hydrolysis in rat brain regions expressing high levels of mGlu5 (hippocampus and striatum) but had no effect in the rat cerebellum, a region primarily expressing mGlu1 receptors.

MPEP [2-methyl-6-(phenylethynyl)pyridine; Fig. 11.14] was discovered following structural modification of SIB-1893, in which the alkenyl linker group was replaced by an alkyne [202]. It is approximately 10 times more potent than either SIB-1757 or SIB-1893, displaying an IC_{50} value of 32 nM in human mGlu5a-expressing cells and 15 nM against 3,5-DHPG-stimulated phosphoinositide hydrolysis in the neonatal rat hippocampus. MPEP blocked constitutive mGlu5 activity in cells overexpressing recombinant mGlu5 receptors, suggesting an inverse agonist mode of action [203]. The binding site for MPEP within the heptahelical domain of mGlu5 was unambiguously identified through a series of elegant experiments utilizing both mGlu1/5 chimeric receptors and mGlu5 single-amino-acid mutants [203, 204]. From these, it appears that MPEP binds within a pocket formed by residues projecting from transmembrane-spanning domains III, V, VI, and VII.

MPEP was initially found to be highly selective for mGlu5 receptors, with high micromolar concentrations producing no statistically significant effects across other cloned mGlu or iGlu receptor subtypes [202]. Subsequent accounts, however, have shown that high concentrations of MPEP (above 10 μ M) can antagonize native NMDA receptor function [205] and potentiate mGlu4 receptors [192]. The NMDA antagonist property of MPEP may at least be partially responsible for observed neuroprotective properties of this molecule [205, 206]. More recently, MPEP has been reported as a relatively potent inhibitor of norepinephrine uptake ($IC_{50} \sim 3 \mu$ M [207]), monoamine oxidase A (MAO_A, $IC_{50} = 8 \mu$ M [197]), and cytochrome P₄₅₀ isoform CYP1A2 (cytochrome P₄₅₀1A2) ($K_i = 0.4 \mu$ M [208]). Nevertheless, owing to its excellent potency, good (though not absolute) pharmacological selectivity, and functional

antagonist activity at mGlu5 receptors following systemic administration [202], MPEP remains a highly valuable tool for evaluating mGlu5 receptor function under physiological and pathophysiological conditions. It has been widely studied as an effective treatment in models of anxiety [90, 209–212], pain [34, 213–221], neuroprotection [205, 206, 222–225], Parkinson's disease [226–229], Huntington's disease [102], and drug abuse [230–233]. The discovery, pharmacology, and activity of MPEP in various disease models have been reviewed [234–237].

In an effort aimed at improving the biochemical selectivity and aqueous solubility of MPEP, 3-[(2-methyl-1,3thiazol-4-yl)ethynyl]pyridine (MTEP; Fig. 11.14) was discovered [197]. MTEP displays mGlu5 binding affinity ($K_i = 16$ nM), functional antagonist potency ($IC_{50} = 5$ nM), and receptor occupancy (median effective dose $ED_{50} = 1$ mg/kg i.p.) comparable to MPEP but with significantly reduced NMDA antagonist ($IC_{50} > 100$ μ M) and MAO_A inhibitory ($IC_{50} = 30$ μ M) activity. More extensive profiling demonstrated excellent selectivity versus other mGlu and iGlu receptor subtypes ($IC_{50} > 10$ μ M) including mGlu4, where it was inactive as a potentiator at concentrations up to 100 μ M [238]. MTEP was also inactive against a broad panel of CNS receptors when screened at 10 μ M [238]. MTEP has a lower brain-to-plasma ratio than MPEP and when dosed systemically is slightly more potent than MPEP in decreasing conditioned responding in the fear-potentiated startle model in rats (MTEP $ED_{50} = 1$ mg/kg i.p. vs. MPEP $ED_{50} = 5$ mg/kg i.p.). MTEP is active acutely in the Geller–Seifter conflict test at doses (3 and 10 mg/kg i.p.) that correspond to mGlu5 receptor occupancy of greater than 90%. At acute doses up to 30 mg/kg, no motor impairment (rotarod) was observed. Curiously, the anxiolytic efficacy of MTEP in the Geller–Seifter model was abolished following three daily (3-mg/kg i.p.) doses. This is in contrast to the maintained anxiolytic activity in the Vogel conflict model for either MPEP [211] or MTEP [239] following 7 days repeat dosing. MPEP also maintained efficacy in the olfactory bulbectomized rat model of depression following repeat dosing for 14 days. The anxiolytic effects of either MPEP [212] or MTEP [239] were not reversed by flumazenil, indicating a GABA-independent mechanism of anxiolysis. However, the anxiolytic (neuropeptide Y) effects of MPEP could be fully blocked by an intra-amygdala dose of the NPY1 (neuropeptide Y) antagonist BIBO 3304 [(R)-N-[[4-(aminocarbonylaminoethyl)phenyl]-methyl]-N²-(diphenylacetyl)-arginamide trifluoroacetate] indicating a role for amygdala NPY signaling in the anxiolytic effects of mGlu5 antagonists. The combination of non-efficacious doses of both MTEP and diazepam resulted in full anxiolytic efficacy, suggesting a possible synergy between mGlu5 antagonist and GABA_A agonist mechanisms [239]. Additional diarylalkynes related to MPEP and MTEP have recently been described [240, 241] as have a number of useful radioligands [197, 242–244].

A number of non-alkyne-containing mGlu5 receptor antagonists have been recently described [245–247]. 2-(3-Methoxy-4-pyridin-2-yl-phenyl)benzoxazole (compound **4**, Fig. 11.14) was described as a potent ($IC_{50} = 41$ nM, $K_i = 159$ nM) and orally bioavailable ($F = 100\%$) mGlu5 antagonist. However, this analog and others from the series demonstrated only modest receptor occupancy following intraperitoneal administration at 10 mg/kg and were inactive in the fear-potentiated startle model of anxiety. 1,3-Diaryl-5-membered ring heterocycles exemplified by 3-fluoro-5-(5-pyridin-2-yl-tetrazol-2-yl)benzonitrile (compound **5**, Fig. 11.14) are potent and selective mGlu5 antagonists (mGlu5 $IC_{50} = 70$ nM, $K_i = 190$ nM) [246]. Compound **5** displays excellent rat bioavailability ($F = 100\%$), receptor occupancy ($ED_{50} = 3$ mg/kg p.o.), and efficacy in the fear-potentiated startle

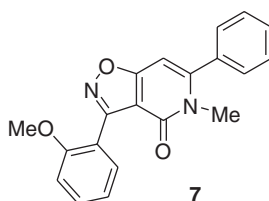


Figure 11.15 mGlu7 allosteric antagonist.

model of anxiety ($ED_{50} = 5.4 \text{ mg/kg p.o.}$); however, apparent tolerance to the anxiolytic effect of **5** in this model was observed, and this compound was also found to be a relatively potent inhibitor ($IC_{50} = 3.8 \mu\text{M}$) of CYP1A2 in vitro [247]. An effort to reduce the CYP1A2 activity of compounds related to **5** led to the discovery of the more highly optimized variant 3-fluoro-5-[5-(5-methyl-1*H*-imidazol-2-yl)-tetrazol-2-yl]benzonitrile (compound **6**, Fig. 11.14: mGlu5 $IC_{50} = 77 \text{ nM}$; $K_i = 19 \text{ nM}$; $F = 22\%$; 97% mGlu5 receptor occupancy at 10 mg/kg, i.p. ; Cyp1A2 $IC_{50} = 70 \mu\text{M}$).

11.3.8 mGlu7 Allosteric Antagonists

Patent applications claiming mGlu7 receptor antagonists have appeared [248, 249]. In particular, 3-(2-methoxy-phenyl)-5-methyl-6-phenyl-5*H*-isoxazolo[4,5-*c*]pyridin-4-one (compound **7**, Fig. 11.15) is reported to antagonize the L-AP4- (Fig. 11.6) induced calcium response in CHO cells expressing recombinant mGlu7 receptors with an IC_{50} value of 7.65 nM .

11.4 FUTURE PERSPECTIVES

Compared to the early 1990s when the first cloned receptor was elucidated, much progress has been made on the pharmacology of mGlu receptors. Advances in the discovery of new agents now include many nanomolar potent compounds with relatively high mGlu receptor selectivity. This includes group I (mGlu1/5) and group II (mGlu2/3) selective orthosteric agonists and antagonists. Some of these compounds (i.e., DCG-IV, LY354740, LY341495) have been useful as high-affinity radioligands, in performing receptor modeling, in investigating mGlu receptor expression levels and receptor regulation, and in determining the relative affinities of compounds for mGlu receptor binding sites in cells expressing cloned receptors and in the rat brain. A number of noncompetitive allosteric antagonists for specific receptor subtypes have now been described and represent great new tools for target validation studies to drive potential future clinical investigations (e.g., CPCCOEt or JNJ16259685 for mGlu1 and MPEP for mGlu5). These agents have also been useful for investigating critical receptor-ligand interactions and have enhanced the understanding of mGlu receptor structure and function. Systemically active pharmacological tools (e.g., LY354740, LY379268, MPEP) have been described that have enabled the exploration in animals of the potential therapeutic applications of mGlu receptor ligands.

From this work, a number of promising therapeutic targets have now been elucidated and are being pursued. Both orthosteric and allosteric mGlu active

compounds are being optimized as drug candidates and have begun moving into the clinic. The most advanced of these include (1) mGlu2/3 receptor agonists as novel anxiolytics and antipsychotics (clinical information is now known), (2) mGlu5 receptor antagonists for anxiety, pain, and drug abuse (optimized compounds and clinical ligands have been disclosed), (3) mGlu1 receptor antagonists for pain and possibly anxiety or epilepsy (optimized compounds and potential clinically useful ligands have been reported), (4) mGlu2 receptor potentiators for stress-related disorders and psychosis (optimization of compounds have been reported), and (5) earlier but promising targets where tools are beginning to establish the biology and uses (mGlu1 potentiators; various mGlu4, mGlu7, and mGlu8 selective compounds).

In summary, the rapid progress made in mGlu pharmacology is remarkable when compared to other areas of neuropharmacology. In a relatively short period of time (about a decade), there are now many highly potent and subtype selective agents, and some are already advancing into the clinic. In part, this may be driven by the structural heterogeneity of the glutamate binding pocket across subgroups and at the allosteric sites across subtypes, along with new technologies for drug discovery (molecular modeling, screening, transgenic animals). Furthermore, the dominant role of glutamate in the physiology/pathophysiology of the nervous system coupled with the more modulatory role of mGlu receptors makes this a promising area for novel therapeutic applications.

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12

PHARMACOLOGY OF THE GABA_A RECEPTOR

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12.1	Transporters	467
12.2	GABA _A , B, and C Receptors	468
12.3	GABA _A Receptor Subunit Types	469
12.4	Regional Distribution of GABA _A Receptor Subunits in CNS	471
12.5	Subcellular Distribution of GABA _A Receptors	473
12.6	GABA _A Receptor structure	475
12.7	Homo-oligomeric GABA _A Receptors	476
12.8	Hetero-oligomeric GABA _A Receptors	476
	12.8.1 Composed of Two Different Subunit Subtypes	476
	12.8.2 Composed of Three or More Different Subunit Subtypes	477
12.9	GABA _A Receptor Assembly	477
12.10	Trafficking of GABA _A Receptors	478
12.11	Modulation of GABA _A Receptors by Phosphorylation	481
12.12	GABA _A Receptor Pharmacology	483
12.13	Pharmacology of GABA _A Receptors Containing Different α Subunits: Search for Molecular Basis of Anxiety and Hypnosis	483
	12.13.1 Terminology	483
	12.13.2 Determining Selectivity of Modulators	484
	12.13.3 α_1 Subunit	484
	12.13.4 α_2 Subunit	489
	12.13.5 α_3 Subunit	490
	12.13.6 α_4 Subunit	490
	12.13.7 α_5 Subunit	490
	12.13.8 α_6 Subunit	491
12.14	Pharmacology of GABA _A Receptors Containing Different β Subunits	491
12.15	Pharmacology of GABA _A Receptors Containing Different γ Subunits	492
12.16	Pharmacology of GABA _A Receptors Containing δ Subunits	493
12.17	Pharmacology of GABA _A Receptors Containing “Rare” Subunits	494
12.18	Structural Determinants of Receptor Activation	494
12.19	Architecture of Agonist Binding Sites	496

12.20	Transduction of Binding to Gating	496
12.21	The Channel Pore and its Ion Selectivity	498
12.22	GABA _A Receptor Desensitization and Deactivation	500
12.23	Modulation of GABA _A Receptor via Allosteric Binding Sites: Benzodiazepine Recognition Site	501
12.24	Benzodiazepine Binding Site of GABA _A Receptors	508
12.25	Structural Determinants of Allosteric Modulation by Benzodiazepines	509
12.26	Benzodiazepine Binding Sites Composed of Different α - and γ -Subunit Isoforms	511
12.27	Modulation of GABA _A Receptors by Neurosteroids, Anesthetics, Alcohols, and Anticonvulsants	513
12.28	Allosteric Sites Within Transmembrane Domain of Receptor Subunits	518
12.29	Channel Blockers and Their Binding Site	520
12.30	Modulation of GABA _A Receptors via Unidentified Allosteric Sites	521
	References	522

Although the human central nervous system (CNS) is undoubtedly a marvel of implementation of cellular systems, composed of roughly 10^{13} neurons each making thousands of synaptic connections, their functional output can be expressed in terms of a balance between excitatory and inhibitory synaptic activity. Approximately one-third of brain synapses use γ -aminobutyric acid (GABA) as the inhibitory neurotransmitter [1–3]. Experiments carried out during the 1950s and 1960s suggested that GABA has an inhibitory effect in the invertebrate [4–6] and the vertebrate CNS [7–15]. The molecular diversity of type A GABA (GABA_A) receptor subunit genes probably evolved by a process of duplication and translocation of an ancestral gene, leading to modern-day gene clusters distributed on different human chromosomes [16–18].

L-Glutamic acid decarboxylase (GAD) catalyzes the conversion of L-glutamate to GABA via α decarboxylation resulting in the accumulation and storage of GABA within inhibitory neurons [5, 19, 20]. Upon arrival of an action potential at the nerve terminal, the presynaptic membrane depolarizes inducing the opening of voltage-gated Ca^{2+} channels. Influx of Ca^{2+} ions through these channels increases its intracellular concentration and triggers the fusion of synaptic vesicles containing GABA with the presynaptic membrane. When released into the synaptic cleft GABA freely diffuses within the 20-nm synaptic cleft and binds to both post- and presynaptic receptors. Termination of synaptic transmission is achieved by clearing the neurotransmitter by a combination of diffusion out of the cleft and active transport into contiguous neuronal and/or glial cells [21].

Once recaptured by the neuron, GABA can be further transported into synaptic vesicles by vesicular GABA transporters (VGATs) or degraded by the enzyme GABA aminotransferase to succinic semialdehyde [22]. Different plasma membrane transporters are responsible for high-affinity uptake of GABA into neuronal cells. These membrane-bound transport proteins are dependent on the Na^+ transmembrane gradient and may require either Cl^- or K^+ for activity [23–25].

The accumulation of intraneuronal GABA into storage vesicles theoretically increases the potential concentration gradient across the plasma membrane by compartmentalization of releasable GABA, protecting it from leakage and/or intraneuronal metabolism.

12.1 TRANSPORTERS

Uptake experiments performed in PC12 cells transfected with VGAT complementary deoxyribonucleic acid (cDNA) indicate that GABA has a low affinity for VGAT ($K_m = 5.5 \text{ mM}$) [26]. Studies with native brain synaptic vesicles suggest that a common transporter is responsible for the vesicular accumulation of both GABA and glycine [27]. In situ hybridization and immunohistochemical experiments show that VGAT is expressed in GABAergic and glycinergic neurons [26, 28–30], supporting the idea that VGAT is a neuronal vesicular transporter for both GABA and glycine. Some GABAergic terminals are devoid of VGAT immunoreactivity [29, 30], suggesting that other vesicular GABA transporters exist.

Different plasma membrane neurotransmitter transporters are responsible for the active movement of GABA into neuronal cells. Such transmembrane transport proteins are Na^+ - and Cl^- -dependent symporters [23–25]. Four classes of GABA transporters responsible for the high-affinity uptake of GABA have been identified thus far: the GABA transporters GAT1 [31–33], GAT2 [32], and GAT3 [34, 35] and a betaine/GABA transporter BGT1 [36, 37] with specific cellular and subcellular distribution [38–40]. GAT1 is expressed in both neurons and astrocytes [41–45]. In addition, GAT1 is synthesized in glutamatergic pyramidal cells of the hippocampus and cerebral cortex [41, 46]. GAT2 is expressed by arachnoid and ependymal cells [47, 48] and GAT3 is found in glial cells [44, 45, 49].

Experiments performed in LLCPK-1 and MDCK-1 epithelial cells transfected with GAT1 cDNA indicate that GAT1 is present only at the apical domain of the transfected epithelial cell [50]. This localization is the topological equivalent of the nerve terminal [51]. Immunohistochemical studies also indicate that GAT1 localization is restricted to axon terminals [39, 47].

GABA transporters have 12 transmembrane (TM) domains linked by hydrophilic loops with the amino and carboxyl termini residing inside the cell [52–54]. Mutagenesis studies suggest that the highly conserved TM1 and TM3 are important for the interaction of the transporters with their substrates [55–60]. TM2 seems to play a role in the formation of oligomers during biosynthesis and targeting to the plasma membrane [61].

Although the main function of neuronal GABA transporters is the clearance of GABA from the synaptic cleft, the transporters also appear to be involved in brain development. The blockade of GAT1 activity in early postnatal hippocampal slices leads to a marked enhancement of GABA_A receptor-mediated spontaneous oscillations of neuronal populations or “giant depolarizing potentials” [62]. These synchronous network events seem to be involved in the maturation of neuronal circuits [63–65]. Whereas GAT2 may play an important role in brain development, in adult brain GAT2 participates in metabolic processes together with BGT1 and is involved in transport of GABA across the blood–brain barrier [66, 67].

The function of GABA transporters can be modulated by Ca^{2+} /calmodulin-dependent protein kinase, protein kinase C (PKC), and cAMP-dependent protein kinase A (PKA) [68–70]. PKC activation markedly increases the activity of GAT1 when it is expressed in *Xenopus* oocytes, and this effect is associated with a shift in GAT1 subcellular localization from intracellular vesicles to the plasma membrane [70, 71]. Site-directed mutagenesis studies reveal that the redistribution of this GABA

transporter is dependent on the presence of a leucine zipper motif in its second TM domain and the level of syntaxin expression [70, 71].

Inhibition of GABA transporters should inhibit the overexcitation observed during stroke and seizures by augmenting inhibition via classical GABA_A and GABA_B receptors. Thus, specific inhibitors of GABA transporters represent a new target class for the development of a novel therapeutic treatment for epilepsy. Similarly, compounds that stimulate the release of GABA by reversing GAT activity might also act as anticonvulsant and neuroprotective agents. The GAT1 inhibitors *cis*-3-aminocyclohexanecarboxylate, CI-966 (1-[2-[bis 4-(trifluoromethyl) phenyl]-methoxy] ethyl]-1,2,5,6-tetrahydro-3-pyridine carboxylic acid), nipecotic acid, NNC 05-711 (1-[2-[[[diphenylmethylene]amino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid), SK&F 89976-A (N-[4,4-diphenyl-3-butenyl]-3-piperidinecarboxylic acid), and tiagabine (Gabitril; (R)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidinecarboxylic acid]) attenuate the convulsive activity in animal models of epilepsy [33, 42, 72]. The rank order of potency of these compounds is NNC 05-711 > tiagabine > SK&F 89976-A > CI-966 [73]. Tiagabine is also efficient in the treatment of complex and refractory myoclonic seizures [72]. Some selective inhibitors of GAT2 and GAT3 are represented by β -alanine, hypotaurine, NNC 05-2045 and NNC 05-2090 [42, 74, 75]. These agents also display anticonvulsive properties [33, 42, 76], consistent with prediction.

12.2 GABA_A, GABA_B, AND GABA_C RECEPTORS

GABA acts via three classes of receptors (GABA_A, GABA_B, and GABA_C) in the vertebrate CNS that differ in their structure, function, and pharmacology. The vast majority of fast GABA responses that are inhibited by bicuculline and picrotoxin and enhanced by benzodiazepines or barbiturates result from the direct activation of an anion channel [4, 5, 9, 77–79] referred to as the GABA_A receptor [80].

A second type of ionotropic GABA receptor (GABA_C) is insensitive to bicuculline, benzodiazepines, and barbiturates [2, 81] (for a review see [82]). While GABA_A receptors are widely distributed in the CNS, the presence of GABA_C receptors is largely restricted to retinal bipolar or horizontal cells across vertebrate species [83–86]. GABA_A receptors are heterooligomeric channel-forming proteins, formed by different subunits that can belong to eight distinct classes (α , β , γ , δ , ϵ , π , θ , and ρ), providing the potential for a huge number of receptor subtypes (Fig. 12.1). In contrast, GABA_C receptors are homooligomeric receptors composed of ρ subunits [103–105]. Although the GABA_C receptor terminology is still used frequently, these receptors are thought to be part of the GABA_A receptor family [87].

Fast-responding GABA receptors are members of the Cys loop ligand-gated ion channel superfamily (reviewed in [87, 106, 107]) comprising nicotinic acetylcholine, GABA_A, GABA_C, glycine, and 5-HT₃ (5-hydroxytryptamine type 3) receptors that possess a characteristic loop formed by a disulfide bond between two cysteine residues. GABA binding to the extracellular domain of GABA_A and GABA_C receptors triggers the opening of an intrinsic chloride-selective ionophore that drives the membrane potential toward the reversal potential for Cl[−] ions (about −80 mV) in neurons. As a consequence, the probability that action potentials will be generated by excitatory neurotransmission is decreased. But GABA_A receptor activation can

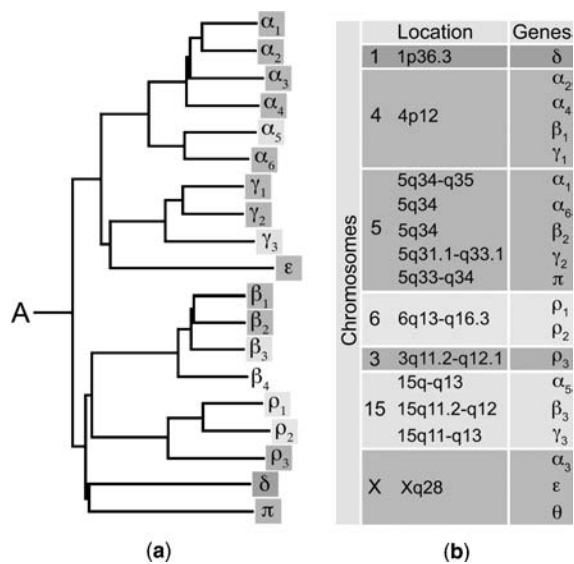


Figure 12.1 (a) Dendrogram depicting relation of amino acid sequences among GABA_A receptor subunits (adapted from [87]) and ancestral gene (A). (b) Localization of genes coding for GABA_A receptor within human genome (from [16–18, 88–102]). (See color insert.)

also induce depolarizing responses, particularly in embryonic neurons. This phenomenon is due to increased intracellular Cl[−] concentration in certain neurons that occurs prior to the expression of the KCC2 (potassium chloride co-transporter type 2) transporter, which is principally responsible for Cl[−] extrusion [108–113]. In adult brain, the GABA_A receptor can also mediate depolarizing responses under certain physiological and pathological conditions that involve intense receptor activation [114].

The slow response to GABA is mediated by GABA_B receptors (for a review see [115]), originally differentiated from GABA_A receptors on the basis of their sensitivity to activation by baclofen (β-parachlorophenyl GABA) [115]. Baclofen is used clinically as a spasmolytic [116, 117], mimicking the agonist effect of GABA in a stereoselective manner. The activation of GABA_B receptors by GABA inhibits neurotransmitter release from different tissue preparations and this effect is not blocked by bicuculline. GABA_B receptors are coupled to Ca²⁺ and K⁺ channels and to adenylyl cyclase via G proteins.

12.3 GABA_A RECEPTOR SUBUNIT TYPES

Twenty related GABA_A receptor subunit genes have been identified in the vertebrate nervous system thus far: 6α, 4β, 3γ, 1δ, 1ε, 1π, 1θ, and 3ρ [87, 101, 118]. A putative β₄-subunit gene, originally identified in chicken [119], has also been described in humans [120]. Figure 12.1 illustrates the seven different subunit sequence families of GABA_A and GABA_C receptors.

Alternative splicing of certain genes produces additional diversity of subunit sequence. There are two splice forms of β₂, β₄, and γ₂ subunits [119, 121–123] that

differ by the presence or absence of a short peptide in the long intracellular loop between TM3 and TM4. Splicing of exon 1 results in two alternative forms of the β_3 subunit [124]. The α_6 subunit is alternatively spliced in approximately 20% of its transcripts in rat brain, causing a deletion at the N-terminus of 10 amino acid residues of uncertain physiological significance [125].

GABA_A receptor subunits are up to 460 amino acids in length and composed of a large N-terminal extracellular domain four TM domains and a large intracellular loop between TM3 and TM4 [126] (Fig. 12.2). The N-terminal extracellular domain carries several potential sites for N-linked glycosylation [127] and two conserved cysteine residues. Binding sites for agonists and ligands of the benzodiazepine binding site are located at the interface between homologous parts of this domain that belong to different subunits. Binding sites for anesthetics, barbiturates, ethanol,

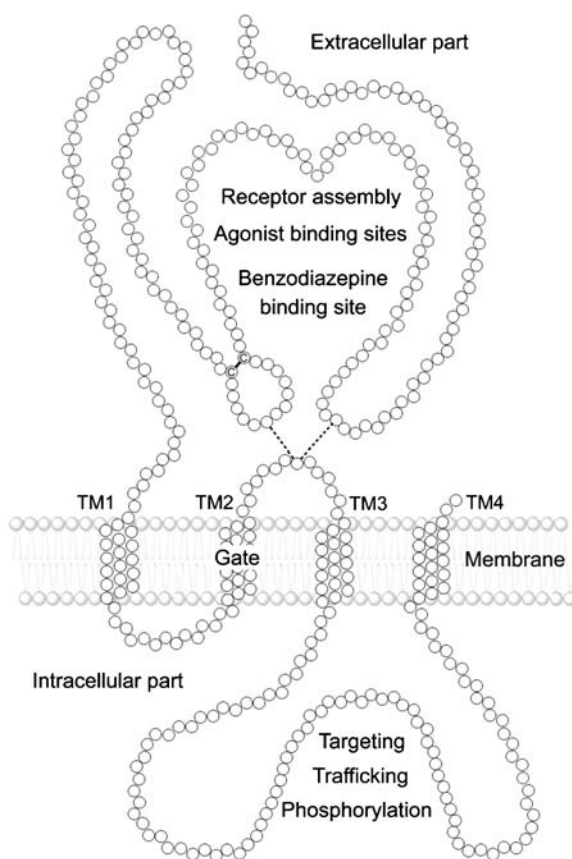


Figure 12.2 Schematic view of GABA_A receptor subunit. The N-terminal part is implicated in receptor assembly and formation of agonist and allosteric modulator binding sites. Different loops of the N-terminal domain are involved in formation of either agonist or benzodiazepine binding sites and signal transduction from ligand binding sites to the channel gate. The intracellular loop between TM1 and TM2 is forming the ion-selectivity filter. The TM2 domains of each subunit line up the channel pore. The loop between TM3 and TM4 is implicated in receptor regulation by secondary-messenger systems, trafficking, and clustering at synaptic membrane.

furosemide, and zinc are located within TM domains. Each subunit contributes to form the channel that is largely formed by residues of TM2 and possibly of TM3 [128–130].

12.4 REGIONAL DISTRIBUTION OF GABA_A RECEPTOR SUBUNITS IN CNS

Subunit isoform distribution in the brain has been studied by *in situ* hybridization [101, 102, 131–135] and immunohistochemical experiments [118, 136–148]. The individual subunits exhibit a distinct but overlapping regional and cellular distribution. The α_1 , β_1 , β_2 , β_3 , and γ_2 subunits are distributed with minor differences throughout the whole brain [148–150], whereas α_2 , α_3 , α_4 , α_5 , α_6 , γ_1 , and δ subunits are more confined to certain brain areas [147, 148].

The α_1 subunit is the most abundant and widely distributed in the brain. It exhibits lesser expression in the striatum, reticular thalamic nucleus, and internal granular layer of the olfactory bulb [146]. The second most abundant subunit is α_2 , which is preferentially located in forebrain areas [146, 148]. The highest α_2 immunoreactivity is observed in the olfactory bulb, striatum, nucleus accumbens, septum, dentate gyrus, amygdala, and hypothalamus and α_2 is less expressed in the thalamus (except reticular nucleus), midbrain, and brain stem areas. Colocalization of α_1 and α_2 with β_2 - and β_3 -subunit immunoreactivities is observed in most hypothalamic areas, raising the possibility of a preferential formation of $\alpha_{1/2}\beta_{2/3}$ receptors [141].

The α_3 subunit is strongly expressed in the glomerular and external plexiform layers of the olfactory bulb, the inner layers of the cerebral cortex, the reticular thalamic nucleus, and the zonal and superficial layers of the superior colliculus, the amygdala, and cranial nerve nuclei [146]. In the raphe nuclei the vast majority of serotonergic neurons express a strong α_3 -subunit immunoreactivity but they are devoid of α_1 -subunit staining and only a small population of serotonergic neurons coexpress both subunits [151, 152], although expression patterns of subunits overlap in certain brain regions. In the medial septum–diagonal band of Broca complex about 85% of neurons containing choline acetyltransferase express the α_3 but not the α_1 subunit. In contrast, 45–60% of parvalbumin-immunoreactive GABAergic neurons express both α_1 and α_3 subunits in various subnuclei of the medial septum–diagonal band of Broca complex, whereas most of the remaining parvalbumin positive neurons have intensive α_1 -subunit immunoreactivity [151, 152]. The α_3 subunit, however, is associated not only with serotonergic or cholinergic neurons but also with noradrenergic and dopaminergic neurons in the brain stem [151, 152]. These data indicate that serotonergic and GABAergic neurons express different α subunits, suggesting that they possess distinct subtypes of GABA_A receptors. The α_4 subunit is strongly expressed in the thalamus, dentate gyrus, olfactory tubercle, and basal ganglia [153]. The α_5 -subunit immunoreactivity is strongest in Ammon's horn, the olfactory bulb, and the hypothalamus [146]. The most striking example of subunit localization is illustrated by the α_6 subunit, which is exclusively expressed in granule cells of the cerebellum and the cochlear nucleus [146]. Colocalization of α_1 - and α_6 -subunit messenger ribonucleic acids (mRNAs) is found in about 50% of granule cells while 25% of these cells contain either the α_1 or α_6 subunit exclusively [154].

The β_2 subunit is perhaps the most widely distributed β subunit in the CNS whereas β_1 and β_3 subunits are less abundant [149]. The relative distribution for β_1 is hippocampus > cortex > cerebellum; for β_2 it is cerebellum > cortex > hippocampus [155, 156]. The β_1 subunit is also located in the neostriatum and inferior olive [146]. The ventral lateral geniculate nucleus contains β_1 , β_2 , and β_3 subunits as well as α_2 , α_3 , and γ_2 subunits [135, 146]. Strong diffusely located staining for the β_3 subunit is observed in the striatum, nucleus accumbens, and olfactory tubercle. The β_3 subunit is presumably colocalized with α_2 , α_4 , and δ subunits on dendrites of the medium spiny neurons. The β_2 subunit is mostly restricted to interneurons of these areas [147].

With regard to γ subunits, γ_2 is the most widely distributed throughout the CNS, whereas γ_1 and γ_3 are relatively scarce [150, 157]. The γ_1 subunit is the least abundant and is specifically distributed in the nervous system. The highest levels of γ_1 immunoreactivity are observed in the globus pallidus, ventral pallidum, entopeduncular nucleus, and substantia nigra pars reticulata, areas innervated by the striatum [146]. The γ_1 subunit is also found in the central and medial amygdaloid nuclei and the inferior olive [147]. The γ_3 subunit is expressed in most brain areas with low abundance, and the highest levels of γ_3 immunoreactivity are in substantia nigra pars compacta and the ventral tegmental area located on numerous presumptive dopaminergic neurons [147, 152].

The δ subunit displays strong but diffuse staining in the striatum, nucleus accumbens, and olfactory tubercle, where it is presumably colocalized with α_2 , α_4 , and β_3 subunits on dendrites of medium spiny neurons [147]. The δ subunit is colocalized with the α_4 subunit in the thalamus, striatum, outer layers of the cortex, dentate molecular layer, and in neonatal hippocampus [158–161] and with the α_6 subunit in the cerebellum [146]. In the neostriatum the δ subunit is colocalized with α_2 , α_4 , β_1 , and β_3 subunits [146].

In situ hybridization studies indicate that the ϵ -subunit is predominantly expressed by neurons located in septal and preoptic areas, various hypothalamic nuclei, amygdala, and thalamus [118, 144, 145]. The ϵ -subunit mRNA is detected in areas containing cholinergic (basal nucleus), dopaminergic (substantia nigra compacta), serotonergic (raphe nuclei), and noradrenergic (locus ceruleus) neurons [118, 144, 145].

The θ subunit [101] seems to be expressed in hypothalamus, amygdala, hippocampus, substantia nigra, dorsal raphe, and locus ceruleus [102, 144]. The θ subunits show overlapping expression patterns with ϵ subunits throughout the brain, especially in the septum, preoptic areas, various hypothalamic nuclei, amygdala, and thalamus, as well as in monoaminergic groups of neurons [144]. In this regard, in situ hybridization and immunohistochemical studies indicate that α_3 , θ , and ϵ subunits present an overlapping distribution in neurons of the dorsal raphe and the locus ceruleus, but θ mRNA is not detected in cholinergic cell groups [144]. These data are consistent with the notion that novel GABA_A receptor subunits may regulate neuroendocrine and modulatory systems in the CNS [118, 144].

The π subunit is detected in several peripheral human tissues as well as in the brain (hippocampus and temporal cortex) and is particularly abundant in the uterus [94]. The detailed regional distribution of the π subunit is unknown. The π subunit can coassemble with α , β , and γ subunits and confer unique ligand and electrophysiological properties to the resulting receptors [94, 162].

The ρ subunits seem to be preferentially expressed in the retina. Immunohistochemical studies of the retina reveal a staining pattern restricted to the terminals of bipolar cells in the inner plexiform layer that does not overlap with α - or β -subunit staining [163–166]. However, ρ -subunit mRNA is also present in the superior colliculus, dorsal lateral geniculate nucleus, and cerebral Purkinje cells [86, 168]. In addition, bicuculline-resistant and baclofen-independent effects of GABA occur in cerebellum [89, 169], superior colliculus [170, 171], amygdala [172], hippocampus [161, 173, 174], dorsal geniculate cells [175], and spinal cord [176]. This indicates that ρ subunits may be present in many regions of the CNS and are more prevalent than initially suspected.

12.5 SUBCELLULAR DISTRIBUTION OF GABA_A RECEPTORS

GABA_A receptors are widely distributed at inhibitory synapses on dendrites and neuronal cell bodies but they are also found at presynaptic, perisynaptic, and extrasynaptic sites (Fig. 12.3). To ask whether receptors occupying different locations have different functions, it is necessary to explore the subcellular distribution of

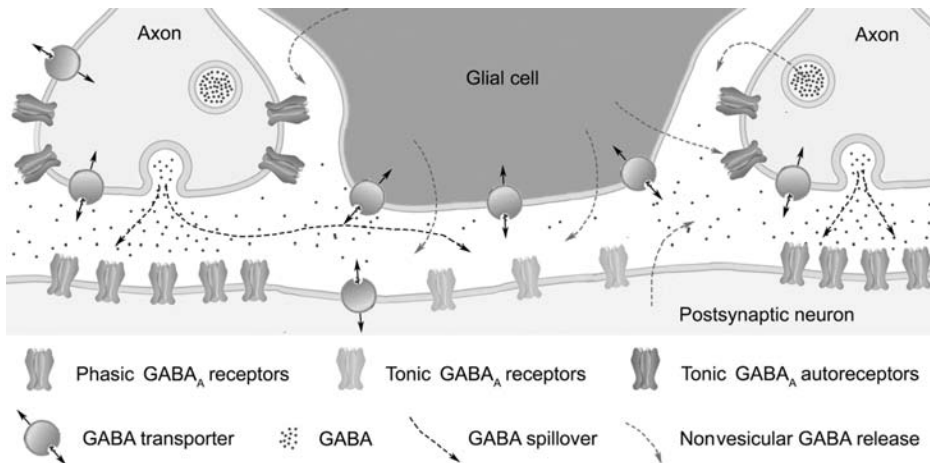


Figure 12.3 Schematic representation of GABAergic synapse. Concentration of GABA in the synaptic cleft is regulated by release, diffusion, and uptake mechanisms. Quantally released GABA can escape from synaptic cleft (GABA spillover) and potentially reach receptors located on extrasynaptic membranes, neighboring presynaptic terminals, and neighboring synapses. Transmitter spillover is thought to underlie both slow-rising GABA_A receptor-mediated IPSCs and part of the tonic GABA_A currents. GABA can also be released via a nonvesicular mechanism by neurons and glia. Once released, GABA can reach target receptors on presynaptic terminals, axon, and somatodendritic compartments. Several action potential-independent mechanisms have been proposed to mediate amino acid neurotransmitter release like reversed transport, stretch-activated anion channels, and P₂X₇ receptor-triggered release. However, blockade of the GABA transporter GAT-1 has no effect on the tonic current, whereas blockade of GAT-3 increases the current. Additional experimental verification of these nonvesicular release hypotheses suggested that GABA might in fact be released from either Golgi cell terminals or astrocytes through a nonvesicular Ca²⁺ independent mechanism. (See color insert.)

different GABA_A receptor subunits. Whereas immunohistochemical studies indicate that the majority of GABA_A receptors at the postsynaptic membrane are composed of α , β , and γ subunits [137–139, 157, 177–182], individual subunits exhibit a cell-specific subcellular distribution [181, 183–187]. For instance, cerebellar granule cells express α_1 , α_6 , β_2 , β_3 , γ_2 , and δ subunits that can form four to six distinct GABA_A receptor subtypes.

The α_1 , α_6 , β_2 , β_3 , and γ_2 subunits are mainly located in GABAergic Golgi synapses and only a small proportion is present at extrasynaptic sites. In contrast, δ subunits are present exclusively in extrasynaptic dendritic and somatic membranes [186]. Receptors composed of $\alpha_6\beta_X\delta$ subunits exhibit a 50-fold higher affinity for GABA than $\alpha_1\beta_X\gamma_2$ receptors [188, 189]. In addition, δ -containing receptors exhibit a smaller single-channel conductance and a much longer open time and do not desensitize after the prolonged presence of GABA as compared with $\alpha_1\beta_X\gamma_2$ receptors [189–191]. The kinetic characteristics and the exclusive extrasynaptic localization of $\alpha_6\beta_X\delta$ receptors suggest that these receptors mediate tonic inhibition in cerebellar granule cells [186, 191–195]. Phasic inhibition of granule cells is due to the transient activation of synaptic $\alpha_6\beta_X\gamma_2$ and/or $\alpha_1\beta_X\gamma_2$ receptors [186, 187, 189, 194].

There are two GABA_A receptor populations in CA1 pyramidal cells—synaptic and extrasynaptic receptors. Synaptic receptors generate rapid spontaneous inhibitory currents (IPSCs). In contrast, extrasynaptic receptors, which are predominant in excised patch preparations, mediate slow responses to brief GABA applications [194, 196, 197]. Receptors composed of $\alpha_4\beta_X\delta$ subunits exhibit properties similar to $\alpha_6\beta_X\delta$ receptors [198] and can, at least partially, be responsible for extrasynaptic tonic inhibition in these cells [195, 199]. Receptors composed of $\alpha_5\beta_X\gamma_2$ subunits are found at extrasynaptic sites in the hippocampus, probably mediating tonic inhibition [200]. The role of these extrasynaptically located GABA_A receptors mediating tonic inhibition in cerebellar granule cells alter the gain of transmission of rate-coded sensory information. Tonic inhibition can be modulated pharmacologically by changes in GABA release and uptake and GABA_A receptor modulators such as ethanol and neurosteroids [189, 197, 201, 202].

Although GABA_A receptors are mainly located in the postsynaptic membrane, presynaptic inhibition appears to be mediated by these receptors as reported in early work based upon pharmacological analysis [203–205], which was confirmed and extended using modern techniques [206–210]. Activation of presynaptic GABA_A receptors at axo-axonic synapses induces depolarization and inhibition of neurotransmitter release. The precise mechanism by which depolarization mediated by presynaptic GABA_A receptors affects transmitter release is not known; however, two main hypotheses have been proposed [211]. First, depolarization can affect transmitter release by acting on other ion channels at or very close to the release site. Thus, depolarization-induced inactivation of voltage-gated Na^+ and/or Ca^{2+} channels leads to a reduction in the amplitude of action potentials and a decrease of Ca^{2+} influx, reducing the probability of neurotransmitter exocytosis. Moreover, depolarization can decrease the driving force for Ca^{2+} influx immediately after the action potential, contributing to the reduction in exocytosis [212]. Second, activation of GABA_A receptors located at a distance from the release sites interfere with the propagation of the action potential into the presynaptic terminal by causing a decrease in membrane resistivity [213, 214]. Although primarily based on computer

simulations, this shunting hypothesis has been supported by experimental evidence from large unmyelinated axons in crayfish [215].

Subunit composition of presynaptic GABA_A receptors in symmetric synapses is generally similar to that of postsynaptic receptors [216]. GABA_A receptors in symmetric synapses are distributed along the entire postsynaptic membrane, whereas in asymmetric synapses the receptors are clustered at the edge of the presynaptic specialization. Thus in the inhibitory synapses GABA_A receptors are localized precisely opposite the GABA release sites of synaptic boutons, whereas in excitatory synapses they are located close to but excluded from the central active zone [216]. At presynaptic inhibitory boutons of crayfish neuromuscular junction, which represents another type of asymmetric synapse, GABA_A autoreceptors are composed of at least the α_3 and $\beta_{2/3}$ subunits [217].

Collectively the results indicate that the interaction of GABA with the receptive target neuron occurs at focal synaptic, perisynaptic, and extrasynaptic sites and that the number and subunit subtype composition of GABA_A receptors present in different populations of synapses may have profound influences on the characteristics of neurotransmission [218–220].

12.6 GABA_A RECEPTOR STRUCTURE

GABA_A receptor subunits share amino acid sequence similarity with nicotinic acetylcholine receptor (nAChR) subunits. Electron optical diffraction of the muscle nAChR receptor in *Torpedo* electric organ (a specialized tissue with exceptionally high receptor density) shows that its three-dimensional structure is that of a pentamer, with the ion channel located in the center of a rosette of five membrane-spanning protein subunits [221–224]. Structural analyses of GABA_A receptors are more complex since no such rich receptor sources exist. Electron microscopic image analysis of GABA_A receptors purified from pig brain cortex indicates a pentameric structure with a central pore [225].

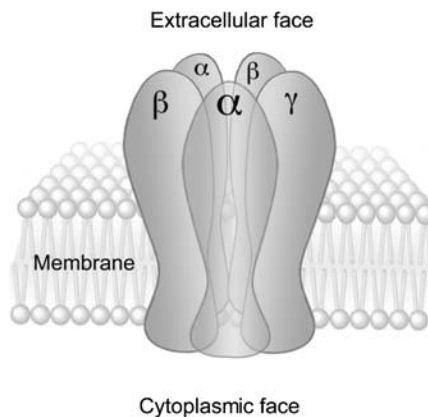


Figure 12.4 Schematic representation of $\alpha\beta\gamma$ GABA_A receptor in postsynaptic membrane. (See color insert.)

Several lines of evidence support a pentameric structure for the GABA_A receptor. Hydrodynamic estimates of the size of GABA_A receptors in solution, either native [226] or $\alpha_1\beta_3\gamma_2$ recombinant receptors [227], are consistent with the molecular weight of a pentamer. Furthermore, the integral ratios of the subunits combined in several forms of functional recombinant receptors, as determined by diverse methods, fit best in each case with total of five subunits [227–231]. Analysis of receptors formed by linked subunits also indicates a pentameric stoichiometry [229, 232, 233] (Fig. 12.4).

Whereas estimates suggest that more than 151,887 GABA_A pentameric receptor subtypes with distinct subunit composition can be formed, at least theoretically [234], some neurons express appreciable quantities of only 2 or 3 different types of subunits and not all subunits can assemble efficiently [107].

12.7 HOMO-OLIGOMERIC GABA_A RECEPTORS

Recombinant expression studies indicate that some of the GABA_A receptor subunits can form homo-oligomers with efficiencies of assembly that vary depending upon the subunit subtype. Homo-oligomeric GABA_A receptors composed of ρ subunits are highly expressed [95, 103–105, 235, 236] while homo-oligomeric receptors containing murine β_1 or β_3 and human γ_{2L} subunits exhibit lower expression levels [237–241].

Interestingly, channels formed by murine or rat β_1 [239, 242] or β_3 subunits [240] can open spontaneously in the absence of GABA and openings are inhibited by the channel blocker picrotoxin. This effect is a function of species since it is not observed with human or bovine β_1 subunits [239, 243, 244].

12.8 HETERO-OLIGOMERIC GABA_A RECEPTORS

12.8.1 Composed of Two Different Subunit Subtypes

Different $\alpha\beta$ -subunit combinations are expressed efficiently and form GABA-activated channels in all systems investigated [245–248]. In contrast, the assembly efficiency of receptors containing $\alpha\gamma$ or $\beta\gamma$ ($\alpha_1\gamma_2$ or $\beta_3\gamma_2$) subunits in HEK-293 cells is low [227]. Receptors composed of $\beta_1\gamma_{2S}$, $\beta_2\gamma_{2S}$ and $\beta_3\gamma_{2S}$ subunits are formed in HEK-293 cells with an efficiency similar to that of homo-oligomeric β_{1-3} receptors, but with different pharmacological properties [249, 250]. The subunit combinations $\alpha_1\gamma_2$ or $\beta_2\gamma_{2L}$ are retained within the endoplasmatic reticulum (ER) [237, 238] and seem unlikely to be functional receptors. These subunit combinations might be formed under certain experimental conditions, such as in the presence of suitable chaperone proteins or at high subunit concentrations. The cotransfection of $\alpha_1\epsilon$ -, $\beta_1\epsilon$ -, $\alpha_1\pi$ -, or $\beta_1\pi$ -subunit combinations does not result in the expression of functional channels in HEK cells [94, 251]. It is unknown whether $\alpha\delta$, $\beta\delta$, or $\gamma\delta$ subunits can form GABA_A receptors.

The ρ subunit can form a functional receptor in vivo [252]. The ρ subunits can also assemble with γ_2 subunits and possibly with glycine receptor subunits to form functional receptors in the retina [253–255].

12.8.2 Composed of Three or More Different Subunit Subtypes

Most GABA_A receptors are composed of α , β , and γ subunits [106, 107, 256]. These subunits can form receptors comprising $(2\alpha/2\beta/\gamma)$, $(2\alpha/\beta/2\gamma)$, or $(\alpha/2\beta/2\gamma)$ assuming a pentameric structure. The most abundant GABA_A receptor in adult mammalian brain is of the $\alpha_1\beta_2\gamma_2$ subtype [106, 107] (Fig. 12.5).

The existence of three identical subunits in the same receptor molecule, for example, $(3\alpha/\beta/\gamma)$, $(\alpha/3\beta/\gamma)$, and $(\alpha/\beta/3\gamma)$ receptors, is not supported by immunoprecipitation [227], electrophysiological [228, 230], and fluorescence energy transfer [231] studies.

Receptors containing two different α - or β -subunit isoforms can assemble and exhibit properties distinct from those receptors containing a single subunit isoform [245, 246, 257–263]. Receptors composed of α_1 , β_1 , the long splice variant of γ_2 , and δ ($\alpha_1\beta_1\gamma_{2L}\delta$) or α_1 , β_3 , γ_3 , and π ($\alpha_1\beta_3\pi$ and $\alpha_1\beta_3\gamma_3\pi$) subunits can also be formed and exhibit properties distinct from $\alpha_1\beta_1\gamma_{2L}$ or $\alpha_1\beta_1\delta$ receptors [190, 261, 264] and from $\alpha_1\beta_3$ or $\alpha_1\beta_3\gamma_3$ receptors [265].

Although experiments investigating the expression of five different subunits have been performed in *Xenopus* oocytes, the results obtained were difficult to interpret [246]. This is not surprising because a variety of different receptor subtypes composed of three, four, or five different subunits could have been formed from five different subunits coexpressed in an oocyte, and all could have contributed to the chloride current measured in these cells. This problem could be addressed by linking multi-subunits using gene fusion, as has already been applied successfully to GABA_A receptors [232, 233, 263, 266].

12.9 GABA_A RECEPTOR ASSEMBLY

GABA_A receptor assembly occurs within the ER [267–270]. Distinct subunit subtypes may confer functionality such as sensitivity to modulation by endogenous ligands such as neurosteroids [271–274], second-messenger systems [270, 275], subcellular localization [237], or long-term differences in the regulation of surface expression [237, 238]. Many neurons express multiple receptor subunit mRNAs [107, 133], suggesting the presence of cellular mechanisms for differential receptor assembly.

The correct arrangement of subunits around the pore depends on assembly constraints conferred by the interfaces of neighboring subunits (Fig. 12.5). The presence of multiple assembly signals in the same subunit can lead to a differential interaction with other subunits and can permit the construction of different GABA_A receptors. Individual subunits are not committed to a particular receptor subtype but can function as universal building blocks in the generation of diverse receptor compositions [107].

Residues 54–68 in the (–) interface of the α_1 are important for the assembly with β subunits [279–281]. Residues 80–100 in the (+) interface of the α_1 subunit are important for the assembly with the γ_2 subunit [282]. Residue Q67 [279] is relevant for the assembly of α_1 with β_3 but not with γ_2 subunits. Conversion of a single amino acid in α_1 to that of γ_2 (R66A) is sufficient to alter the assembly profile of the α_1 subunit to that of the γ_2 subunit. The amino acid R66 is required for the assembly of

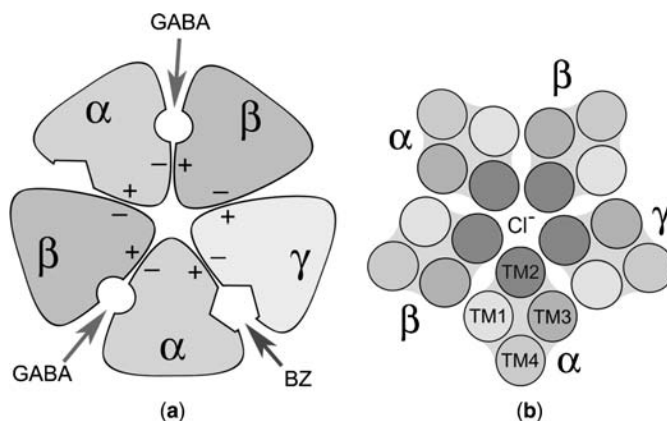


Figure 12.5 Top view of GABA_A receptor. (a) Each subunit contacts two other subunits at two different subunit interfaces. In the homologous nAChR these interfaces have been called principal and complementing [276, 277]. In AChBP the nomenclature “+” and “−” has also been used to denominate these interfaces [278]. (b) Top view of TM part of GABA_A receptor, TM2s of all five subunits contribute to Cl^- channel. (See color insert.)

$\alpha_1\beta_2$ but not $\alpha_1\beta_1$ or $\alpha_1\beta_3$ [283, 284], and two tryptophan residues $\alpha_1\text{W69}$ and $\alpha_1\text{W94}$ are critical for the assembly of the GABA_A receptor pentamer [285].

In the (−) interface of β_2 and β_3 subunits the amino acids 52–66 are critical for the assembly with α_1 subunits [249, 280, 281]. The residues 76–89 located in the (+) interface of the β_3 subunit are important for the assembly with α_1 subunit [286].

The amino acid sequences $\gamma_2\text{67–81}$ [280] and $\gamma_3\text{70–84}$ [287] located in the (−) interface and $\gamma_2\text{83–90}$ and $\gamma_2\text{91–104}$ located in the (+) interface [281] are involved in the assembly of α_1 and β_3 subunits. The amino acid $\gamma_2\text{W82}$ residing in the (−) interface is critical for assembly with α_1 and β_2 subunits [288]. Interestingly, the assembly signals in the α , β , and γ subunits [287, 327, 328] overlap with the GABA [289] and benzodiazepine binding sites [288, 290–294], which are formed at subunit interfaces between the α/β and α/γ subunits, respectively.

12.10 TRAFFICKING OF GABA_A RECEPTORS

Receptor trafficking is a rather complicated process that differs depending on cell type and the subunit composition of receptors [295, 296]. Different proteins that interact with GABA_A receptor subunits are believed to regulate the trafficking of receptors to specific subcellular locations (Fig. 12.6).

The clustering of GABA_A receptors at postsynaptic membranes depends on the scaffolding protein gephyrin, but no evidence for an action of gephyrin in trafficking could be found as of this writing. Studies in gephyrin knockout (KO) mice indicate that GABA_A receptors containing α_2 and γ_2 subunits require gephyrin for synaptic targeting [302] and γ_2 subunit KO mice exhibit a loss in synaptic gephyrin immunoreactivity [303] and postsynaptic GABA_A receptors [304]. These results demonstrate that the presence of γ_2 subunits and gephyrin is important for postsynaptic receptor clustering.

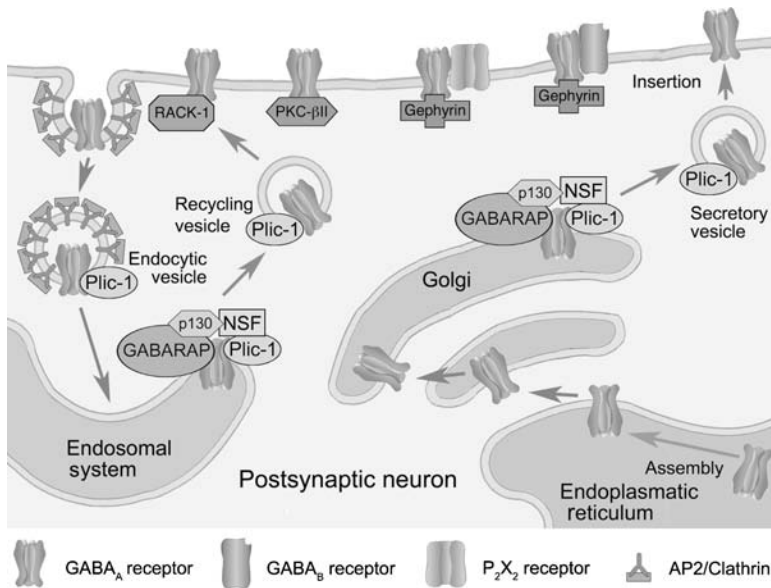


Figure 12.6 GABA_A receptor trafficking to membrane is facilitated by many receptor-associated proteins. GABA_A receptors are assembled in the endoplasmic reticulum. Subsequently competent subunit combinations (e.g. $\alpha\beta$ and $\alpha\beta\gamma$) are transported to the Golgi. In this organelle, assembled GABA_A receptors interact with proteins that facilitate transport to neuronal membrane. The GABA_A receptor γ_2 subunit can interact with GABARAP [314], a protein that interacts with and stabilizes the GABA_A receptor in neuronal membrane and catalytically inactive phospholipase P130 [317] that is able to compete with GABARAP for the same binding site on the γ_2 subunit. Additionally they interact with NSF, a protein that plays a key role in intracellular transport. These multiple protein–protein interactions are believed to facilitate GABA_A receptor intracellular transport. GABA_A receptors also interact directly with the ubiquitin-like protein Plic-1 [307], which stabilizes intracellular receptor pools from the secretory or endocytic pathway that can be recycled and inserted back into membrane. In addition, GABA_A receptors at synaptic sites undergo constitutive dynaminddependent, clathrin-mediated endocytosis. This process is mediated by the interaction of receptor β and γ subunits with α and β adaptins, proteins that are essential for the receptor internalization into clathrin-coated pits. Internalized receptors enter the endosomal transport system, from which they can be returned to the cell surface or degraded. At synaptic sites, GABA_A receptors colocalize with gephyrin, which is believed to play a crucial role in receptor clustering at synapses. In addition to their roles in the secretory pathway, GABARAP, P130, and Plic-1 may also function in the endocytic pathway to mediate and regulate GABA_A receptor trafficking and stability of intracellular receptor pools. GABA_A receptors at cell surface are also intimately associated with several other receptors and signaling proteins, including the functional GABA_B receptor [297], dopamine D₅ [298], ATP-gated excitatory P₂X receptor [299–301], RACK-1, and the β II isoform of PKC [268]. (See color insert.)

Although the results suggest a role for gephyrin in GABA_A receptor clustering, a direct interaction between these two proteins has not been demonstrated. In addition, the postsynaptic localization of α_1 and α_5 subunits in the spinal cord of gephyrin in KO mice is unaffected [305]. Taken together, these observations have stimulated the search for additional proteins important for GABA_A receptor clustering [306–308].

The first protein identified was the GABA_A receptor-associated protein (GABARAP), a 17-kDa polypeptide that interacts specifically with the major intracellular domains of receptor γ_2 subunits [308, 309]. GABARAP is a member of a protein family, which includes the light chain 3 (LC3) of microtubule-associated proteins 1A and 1B, the Golgi-associated adenosine triphosphatase (ATPase) enhancer (GATE-16), and the yeast ortholog Apg8p/Aut7p. Interestingly, GATE-16, LC3, and Apg8p/Aut7p all participate in membrane-trafficking processes [310–312]. Structurally, GABARAP is comprised of an amino-terminal tubulin binding region, which is important for tubulin polymerization, and an ubiquitin-like carboxy-terminal domain, which is capable of binding to the intracellular loop of γ_2 subunit [309, 313].

Overexpression of GABARAP in a mammalian cell line results in increased numbers of clustered GABA_A receptors with modified kinetics [3314]. However, GABARAP immunoreactivity is low in GABAergic synapses and is predominantly localized to intracellular membranes, including the edges of Golgi stacks and a sub synaptic tubulovesicular compartment [315]. Furthermore, GABARAP interacts in intracellular membrane compartments with the *N*-ethylmaleimide-sensitive factor (NSF), a protein involved in membrane fusion events [315]. Expression of recombinant GABARAP cDNA in cultured neurons induces an increase in GABA_A receptor levels at the plasma membrane. These results support a role for GABARAP in the intracellular transport and cell surface targeting of receptors [316].

Yeast two-hybrid screening indicates that GABARAP interacts with p130, a protein structurally related to phospholipase C but lacking the catalytic activity [317]. In addition, GABARAP binding to the GABA_A receptor γ_2 subunit is competitively inhibited by p130. These results, together with the intracellular localization of p130, suggest a role of p130 controlling the membrane transport and assembly of functional GABA_A receptors [317].

Inhibitory neurotransmission can be regulated by the insertion of newly synthesized or recycled GABA_A receptors into synaptic membrane. There is a direct relationship between the number of synaptic GABA_A receptors and the strength of the synapse [318, 319] so modulation of the insertion and/or removal rate of GABA_A receptors into or from the synaptic membrane has a marked effect on the amplitude of mIPSCs (miniature inhibitory postsynaptic currents) [268].

Insulin induces a rapid recruitment of functional GABA_A receptors to postsynaptic plasma membranes, thereby increasing the amplitude of the GABA_A receptor-mediated mIPSCs [320]. In contrast, brain-derived neurotrophic factor (BDNF) produces a reduction of mIPSCs by decreasing the number of postsynaptic GABA_A receptors in cultured hippocampal neurons [321].

The ubiquitin-like protein Plic-1 interacts with the major intracellular loop of the GABA_A receptor α and β subunits and increases the half-life of intracellular receptor pools [307]. Plic-1 has a molecular weight of 67 kDa and contains a ubiquitin-like amino terminus and a carboxy-terminal ubiquitin-associated domain. The Plic-1 immunoreactivity GABA_A receptor is associated with cisternae, the Golgi compartment, invaginations of the plasma membrane, and structures in the vicinity of the postsynaptic membrane [307]. These results indicate that Plic-1 increases the number of GABA_A receptors available for insertion into the plasma membrane [307].

Palmitoylation of cysteine residues is a reversible posttranslational modification that regulates the trafficking and function of different membrane proteins in neurons (for a review see [322–324]). The Golgi-specific zinc finger protein (GODZ) is a palmitoyltransferase that interacts with cysteines in the large cytoplasmic loop of γ_{1-3} GABA_A receptor subunits [325]. Rathenberg et al. [324] demonstrated that the γ_2 subunit is palmitoylated. Reduction of palmitoylation by specific inhibitors, or mutations introduced in the palmitoylation sites of the γ_2 subunit decrease receptor clustering, suggesting that palmitoylation may regulate of GABA_A receptor function of inhibitory synapses.

Several lines of evidence indicate that GABA_A receptors are internalized via clathrin-mediated endocytosis. These include the presence of GABA_A receptors in clathrin-coated vesicles [326, 327] and the interaction of GABA_A receptors with the clathrin adapter protein 2 (AP2) complex, which is critical for membrane protein endocytosis [267, 328]. Furthermore, the β_2 subunit has a dileucine AP2 adaptin binding motif that is important for receptor internalization [329, 330].

12.11 MODULATION OF GABA_A RECEPTORS BY PHOSPHORYLATION

The intracellular domains of GABA_A receptor subunits can be phosphorylated by a variety of kinases at serine, threonine, and tyrosine residues [331] (Fig. 12.7). Studies on recombinant GABA_A receptors have revealed that the major intracellular loops of receptor β and γ subunits can be phosphorylated by a variety of protein kinases, namely cyclic adenosine mono-phosphate (cAMP)-dependent protein kinase (PKA) [275, 336–344], protein kinase C (PKC) [336, 338, 345–349], the tyrosine kinase Src (sarcoma) [335, 350, 351], tyrosine kinase receptor B (TrkB) [352–354], Ca^{2+} /Calmodulin type II-dependent protein kinase (CamKII) [355, 356], and cyclic guanine mono-phosphate (cGMP)-dependent protein kinase [338, 355, 356].

Modulation of GABA_A receptor function by PKA phosphorylation depends on the β -subunit subtype. GABA-mediated currents are inhibited by phosphorylation of Ser409 in the β_1 subunit by PKA; however, phosphorylation of Ser408 and Ser409 in the β_3 subunit(s) by PKA potentiates the GABA response. Activation of PKA does not alter responses from β_2 subunit-containing receptors [349, 356, 357].

PKC phosphorylates the conserved serine residues β_1 S409 and β_2 S410 [336, 348, 349], as does CamKII [348] and cGMP-dependent protein kinase [356]. CamKII phosphorylates residue β_3 Ser383 [356].

The γ subunits are phosphorylated by PKC at residues γ_{2L} S327/ γ_{2S} S327 and γ_{2L} S343 [121, 338, 346]; by CamKII at residues γ_{2L} S327, γ_{2L} S343, and γ_{2L} T348 [355]; and by the prototypic tyrosine kinase Src at residues γ_2 Y365 and γ_2 Y367 [350, 351, 359, 360].

Phosphorylation of GABA_A receptor $\beta_{1/2}$ and $\gamma_{2S/L}$ subunits by PKC inhibits receptor function [361]. Activation of PKC in HEK-293 cells expressing recombinant GABA_A-Rs also induces a reduction in the number of receptors at the cell surface, but the mechanism is independent of receptor phosphorylation [362].

Different signaling pathways control phosphorylation of GABA_A receptors in neurons. The receptor for activated C kinase (RACK-1) and PKC bind independently to adjacent residues in the intracellular domain of the β_1 subunit. Inhibition of RACK-1 binding to β_1 decreases the PKC-mediated phosphorylation of the residue

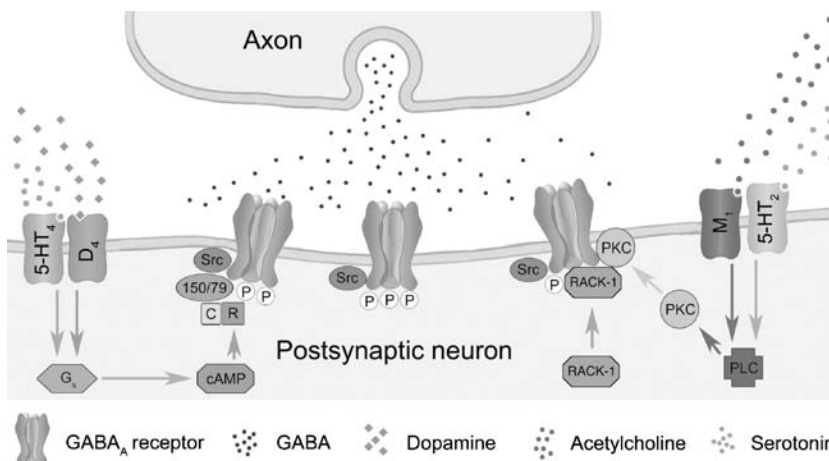


Figure 12.7 GABA_A receptors are intimately linked to G-protein-coupled receptors (GPCRs) via multiple protein kinases and scaffold proteins. GABA_A receptor β and γ_2 subunits are phosphorylated by PKA and PKC upon the activation of corresponding GPCRs for muscarinic M1 [359], dopamine D4 [332], and serotonin 5-HT₂ [333, 334] and 5-HT₄ [268, 335]. Phosphorylation of GABA_A receptor β_1 and β_3 subunits by PKA is dependent upon AKAP150/79, which directly interacts with these receptor subunits and able to bind PKA that is composed of regulatory (R) and catalytic (C) subunits in both active and inactive form. In addition, the GABA_A receptor β_{1-3} and γ_2 -subunits can be phosphorylated by PKC. Upon activation of the appropriate GPCR, PKC-mediated phosphorylation is facilitated through the direct (but independent) interaction of the RACK-1 [358], the β isoform of PKC with the GABA_A receptor β_{1-3} subunits. RACK-1 regulates the function of GABA_A receptors via control of activity of PKC associated with these proteins. The GABA_A receptor γ_2 subunit is also phosphorylated by Src kinase [331], which is also able to bind to the β subunits. Finally, depending on subunit composition of particular GABA_A receptor, phosphorylation can modulate its function in the direction of both inhibition and potentiation. (See color insert.)

β_1 S404 and the inhibitory effect of PKC on GABA-evoked currents [359]. Activation of muscarinic acetylcholine receptors (AChRs) inhibited GABA_A receptor function by a PKC-mediated pathway that depends on the binding of RACK-1 to the GABA_A receptor. These results indicate that phosphorylation of GABA_A receptors by PKC and the regulation of receptor function by PKC-mediated signaling pathways depend on RACK-1 binding to receptors [359]. RACK-1 also participates in the PKC-dependent modulation of GABA_A receptor currents by 5-HT₂ receptors in cortical neurons [363].

A kinase anchoring proteins (AKAPs) often target PKA to its substrates [364]. The neuronal AKAP (AKAP79/150) binds to GABA_A receptors containing the β_1 or β_3 but not the α_1 , α_2 , α_3 , α_6 , β_2 , γ_2 , or δ subunits and is critical for PKA-dependent phosphorylation of the highly conserved β_1 S409/ β_3 S408 residues [365]. These results suggest a mechanism for the PKA-mediated phosphorylation of GABA_A receptor subtypes. Electrophysiological evidence also additionally suggested another AKAP, the scaffold protein Yotiao [366], which may be involved in PKA-mediated modulation of GABA_A receptor function [332].

Activation of tyrosine kinase receptors B (TrkB) by BDNF produces a biphasic temporal modulation of GABA_A receptor function in cultured hippocampal neurons. Early exposure to neurotrophin (5 min after BDNF application) enhances GABA-mediated currents while a late exposure (30 min after BDNF application) inhibits GABA-evoked responses. This modulation parallels an initial increase in PKC-mediated phosphorylation of GABA_A receptors and a subsequent receptor dephosphorylation by the protein phosphatase 2A (PP2A). These results indicate that BDNF dynamically regulates GABA_A receptor function by changing the phosphorylation state of receptors. The mechanism seems to involve a differential recruitment of PKC, RACK-1, and PP2A to GABA_A receptors [353]. The modulation of GABA_A receptor responses by BDNF is age dependent since BDNF potentiates GABA-gated currents of pyramidal neurons isolated from postnatal day 6 rats and inhibits GABA responses on postnatal day 14 [352].

12.12 GABA_A RECEPTOR PHARMACOLOGY

Distinct GABA_A receptor subtypes differ in their channel kinetics, affinity for GABA, rate of desensitization, subcellular positioning, and pharmacology. In the absence of pharmacological tools to specifically and selectively distinguish subunit subtypes, the function *in vivo* of defined receptor subtypes cannot be fully investigated. Therefore, alternative approaches have been employed to address this problem. For instance, specific subunit isoforms can be genetically deleted by creating KO mice, and although such deletions are expected to affect all receptors containing the particular subunit subtype, compensatory upregulation of other subunit subtypes occurs. The knockin approach, wherein a subunit subtype is mutated in order to alter its pharmacological properties, is an alternative strategy that in principle reduces these compensatory changes. Thus, for the α_1 , α_2 , α_3 , and α_5 subunits, replacing histidine-101 with arginine (α_1 H101R, α_2 H101R, α_3 H101R, and α_5 H101R) renders GABA_A receptors insensitive to classical benzodiazepines [367, 368]. A similar approach has been applied to β_2 and β_3 subunits with the introduction of β_2 N265S and β_3 N265S mutations, which render receptors insensitive to general anesthetics [369, 370].

12.13 PHARMACOLOGY OF GABA_A RECEPTORS CONTAINING DIFFERENT α SUBUNITS: SEARCH FOR MOLECULAR BASIS OF ANXIETY AND HYPNOSIS

12.13.1 Terminology

Experts in the field use two different terminologies to describe the effects of benzodiazepine and related therapeutic agents on GABA_A receptor function and on the pharmacodynamic effects of the drugs under investigation. The action of the neurotransmitter GABA (the natural agonist) is blocked by compounds (antagonists; such as bicuculline) that reduce its binding to the GABA recognition site. Benzodiazepines (such as chlordiazepoxide and diazepam) and related chemical entities are known to potentiate the response of GABA_A receptors by shifting the dose–effect

curve for GABA to the left, and this potentiation of the response to the agonist is referred to as positive modulation [11–15, 371–373], and such compounds are positive modulators. Positive modulators almost always shift the dose–effect curve to the left, increasing the potency of GABA for its receptor, but in principle the term would apply to compounds that might increase the maximum response (or efficacy) of GABA. Negative modulators such as β -carboline shift the dose–effect curve for GABA to the right and can also decrease the maximal response (efficacy) to GABA. Null modulators such as flumazenil exhibit little or no modulation of the GABA response but antagonize the binding and action of both positive and negative modulators.

When positive modulators are administered to animals, they exert various pharmacological effects (including sedation, hypnosis, muscle relaxation, and amnesic and anxiolytic activity) and thus can be referred to as behavioral agonists. However, the term agonist should not be used to describe the modulatory actions of these compounds on the GABA_A receptor. Negative modulators are referred to as inverse agonists when administered *in vivo* as they exhibit physiological effects that are generally the inverse of positive modulators, eliciting anxiogenic responses and some even enhancing learning and memory. Null modulators exhibit little or no physiological effects in experimental animals or human subjects but block the effects of positive modulators such as benzodiazepine agonists and negative modulators such as β -carboline inverse agonists.

12.13.2 Determining Selectivity of Modulators

In an attempt to develop an anxiolytic therapeutic agent, an intensive discovery effort was directed toward the design of subtype selective benzodiazepine recognition site ligands. Zolpidem, one such compound, was for a long time considered to be GABA_{A1} receptor selective and has been used in a number of animal studies as standard for the contribution of α_1 -containing receptors to functional responses *in vivo* and *in vitro* systems. However, zolpidem's selectivity *in vivo* has not been established. The strongest evidence for selectivity is based on radioligand binding studies in which it exhibits only a 3.7- to 5.9-fold higher affinity toward $\alpha_1\beta_2\gamma_2$ -containing versus $\alpha_2\beta_2\gamma_2$ -containing receptors, although it does exhibit a much greater separation of affinities with other α -subunit-containing receptor subtypes (see Tables 12.1 and 12.2). There is a fundamental difference between preference and selectivity—a compound is generally considered to be selective for a certain subunit subtype if it exhibits a ≥ 10 -fold higher affinity with respect to the other subtypes. There is insufficient information from functional determinations to come to general conclusions with regard to zolpidem potency, but zaleplon and zopiclone exhibit 2- to 4-fold preference for α_1 - over α_2 -subunit-containing GABA_A receptors (Table 12.2).

12.13.3 α_1 Subunit

The behavioral effects of zolpidem, a GABA_{A1}-preferring positive modulator of GABA action with higher affinity toward $\alpha_1\beta_2\gamma_2$ versus $\alpha_2\beta_2\gamma_2$ receptors, and triazolam, a nonselective positive modulator, were studied in Squirrel monkeys. Flumazenil, which is a nonselective weak partial-positive modulator of GABA action (antagonist of benzodiazepine binding), antagonizes the behavioral effects of both zolpidem and triazolam. This suggests that both zolpidem and triazolam are acting

TABLE 12.1 Binding Affinities (nM) of Benzodiazepine Site Ligands to GABA_A Receptors Containing Different α Subunits as Measured by Radioligand Binding

Compound ^k	$\alpha_{1-6}\beta_x\gamma_2^a$ (Affinity, nM)					
	$\alpha_1\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_3\beta_2\gamma_2$	$\alpha_4\beta_2\gamma_2$	$\alpha_5\beta_2\gamma_2$	$\alpha_6\beta_2\gamma_2$
Chlordiazepoxide ^b	890 ± 250	460 ± 99	980 ± 110	> 10000	620 ± 200	—
Diazepam	12 ± 1 ^b	7 ± 0.5 ^b	33 ± 3 ^b	> 10000 ^b	11 ± 1 ^b	> 10000
Flunitrazepam	3.9 ± 0.5 ^b	1.1 ± 0.3 ^b	5.9 ± 0.7 ^b	> 10000 ^b	1.7 ± 0.5 ^b	> 10000 ^c
Triazolam ^b	0.41 ± 0.01	0.32 ± 0.04	1.5 ± 0.1	> 10000	0.42 ± 0.05	—
Bretazenil	0.22 ± 0.02 ^b	0.37 ± 0.07 ^b	0.43 ± 0.09 ^b	43 ± 2 ^b	0.86 ± 0.22 ^b	> 10000 ^d
Abecarnil ^b	1.3 ± 0.2	2.4 ± 0.3	4.4 ± 0.2	1190 ± 22	5.0 ± 0.3	—
Ro15-1788	1.1 ± 0.04 ^b	1.5 ± 0.1 ^b	1.0 ± 0.1 ^b	240 ± 27 ^b	0.50 ± 0.08 ^b	115 ± 10 ^e
Ro15-4513	4.8 ± 0.1 ^b	7.3 ± 1.2 ^b	2.4 ± 0.4 ^b	5.1 ± 0.6 ^b	0.13 ± 0.01 ^b	6.5 ± 0.6 ^c
Zopiclone	28 ± 3 ^b	64 ± 5 ^b	29 ± 2 ^b	> 10000 ^b	46 ± 8 ^b	> 10000 ^f
Zolpidem	23 ± 5 ^b	110 ± 23 ^b	320 ± 4 ^b	> 10000 ^b	> 10000 ^b	> 10000 ^d
Zaleplon ^f	66 ± 11	830 ± 149	710 ± 160	—	1780 ± 320	> 100000
FG8094 ^g	48.5 ±	22.4 ±	24.5 ±	—	0.45 ±	—
FG8205	0.9 ± 0.2 ^b	2.4 ± 0.2 ^b	1.8 ± 0.3 ^b	440 ± 55 ^b	5.1 ± 1.8 ^b	227 ^d
CGS9895 ^b	0.320.05	1.1 ± 0.2	0.28 ± 0.05	103 ± 8	0.96 ± 0.15	—
CGS8216 ^b	0.17 ± 0.01	0.49 ± 0.09	0.30 ± 0.06	35 ± 11	1.3 ± 0.2	—
CL218872	66 ± 0.3 ^b	720 ± 110 ^b	850 ± 120 ^b	> 10000 ^b	460 ± 13 ^b	> 10000 ^d
Ocinaplon ^h	5367 ± 108	29367 ± 684	5956 ± 53	—	9129 ± 152	—
β-CCM	2.4 ± 0.3 ^b	7.4 ± 1.4 ^b	72 ± 14 ^b	1040 ± 90 ^b	44 ± 6 ^b	2018 ± 190 ^e
β-CCT	0.72 ± ^d	15 ± ^f	18.9 ± ^d	897 ± 131 ^d	111 ± ^d	4745 ± 1045 ^e
DMCM	10 ± 0.7 ^b	13 ± 3 ^b	7.5 ± 0.7 ^b	30 ± 8 ^b	2.2 ± 0.6 ^b	101 ± 11
SL651,498 ⁱ	17 ± 1.5	73 ± 7.0	80.3 ± 10.4	—	215 ± 7	—

L-655,708 ^c	48.5 ± 6	27.4 ± 2.7	24.5 ± 2	—	0.45 ± 0.15	83.2 ± 4.5
L-838,417 ^j	0.79 ± 0.18	0.67 ± 0.24	0.67 ± 0.15	267 ± 19	2.25 ± 0.75	2183 ± 65

^aReceptors were immunoprecipitated or expressed in clonal cell lines.

^bFrom [583].

^cFrom [554].

^dFrom [611].

^eFrom [612].

^fFrom [549].

^gFrom [610].

^hFrom [759].

ⁱFrom [551].

^jFrom [610].

^k*Abbreviations:* Chlordiazepoxide: 9-chloro-5-hydroxy-N-methyl-6-phenyl-2,5-diazabicyclo[5.4.0]undeca-1,6,8,10-tetraen-3-imine; Diazepam: 9-chloro-2-methyl-6-phenyl-2,5-diazabicyclo[5.4.0]undeca-5,8,10,12-tetraen-3-one; Flunitrazepam: 6-(2-fluorophenyl)-2-methyl-9-nitro-2,5-diazabicyclo[5.4.0]undeca-5,8,10,12-tetraen-3-one; Triazolam: 8-chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine; Bretazenil: 9H-Imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylic acid; Abecarnil: isopropyl 6-benzoyloxy-4-methoxymethyl-beta-carboline-3-carboxylate; β -CCM: beta-carboline-3-carboxylate; β -CCE: ethyl-beta-carboline-3-carboxylate; DMCM: dimethoxy-4-ethyl-beta-carboline-3-carboxylate; CL218872: 3-methyl-6-(3-[trifluoromethyl]-phenyl)-1,2,4-triazolo-[4,3-b]pyridazine; Imidazenil: 6-(2-bromophenyl)-8-fluoro-4H-imidazo-[1,5-a]-[1,4]benzodiazepine-3-carboxamide; Loreclezole: (Z)-1-[2'-chloro-2-(2,4-dichlorophenyl)ethenyl]-1,2,4-triazole; Midazolam: 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo(1,5-a)(1,4)-benzodiazepine; Ro15-1788: 4H-Imidazo(1,5-a)(1,4)benzodiazepine-3-carboxylic acid, 8-fluoro-5,6-dihydro-5-methyl-6-oxo-, ethyl ester; Ro15-4513: ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-1,4]benzodiazepine-3-carboxylate; Ro19-4603: t-butyl 5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]thieno[2,3-f][1,4]-diazepine-3-carboxylate; SL-651,498: 6-fluoro-9-methyl-2-phenyl-4-(pyrrolidin-1-yl-carbonyl)-2,9-dihydro-1H-pyrido[3,4-b]indol-1-one; Zolpidem: N,N,6-trimethyl-2-p-toyl-imidazo(1,2-a)pyridine-3-acetamide; Zopiclone: [8-(5-chloropyridin-2-yl)-7-oxo-2,5,8-triazabicyclo[4,3,0]nona-1,3,5-trien-9-yl]-4-methylpiperazine-1-carboxylate; Zaleplon: N-[3-(7-cyano-1,5,9-triazabicyclo[4,3,0]nona-2,4,6,8-tetraen-2-yl)phenyl]-N-ethylacetamide; CGS8216: 2-phenylpyrazolo[4,3-c]quinolin-3(5H)-one; CGS9895: 2,5-dihydro-2-(p-methoxyphenyl)pyrazolo[4,3-c]quinolin-3(5H)-one; L-655,708: (11,12,13,12a-Tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylic acid, ethyl ester; L-838,417: 7-tert-butyl-3-(2,5-difluorophenyl)-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-1,2,4-triazolo[4,3-b]pyridazine; Ocinalon: pyrazolo[1,5-a]pyrimidine

TABLE 12.2 The Potency (nM) and Efficacy of Positive (% Enhancement) and Negative (% Inhibition) Modulators of GABA_A Receptors Containing Different Types of Subunits Measured Using Electrophysiology

Compound	$\alpha_{1-6}\beta_x\gamma_2^a$ (Potency, nM/efficacy, %)					
	$\alpha_1\beta_x\gamma_2$	$\alpha_2\beta_x\gamma_2$	$\alpha_3\beta_x\gamma_2$	$\alpha_4\beta_x\beta_2$	$\alpha_5\beta_x\gamma_2$	$\alpha_6\beta_x\gamma_2$
Chlordiazepoxide ^b	—	—	—	—	—	—
Diazepam ^c	—	—	—	4 ± 9% ^q	+ 134 ± 13	—
	—	—	—	—	—	—
Flunitrazepam ^d	+ 156 ± 10	+ 89 ± 7%	211 ± 19%	—	+ 73 ± 9%	—
	29 ± 11.2,	—	24 ± 10.4,	—	—	—
	+ 100 ± 8.9%	—	+ 100 ± 7.4%	15 ± 11% ^q	101 ± 8% ^q	—
Triazolam	46 ± 10.2, ^d	—	16 ± 1.6, ^d	—	—	—
	+ 81 ± 3.4% ^d	+ 109% ^e	+ 90 ± 2.6% ^d	—	+ 77% ^f	+ 90% ^g
Bretazenil	—	—	—	—	—	—
Abecarnil	+ 51, + 94% ^h	+ 48% ^h	+ 145% ^h	+ 58% ^h	+ 57 ⁱ	—
	—	—	—	—	—	—
Ro15-1788 ^g	+ 74 ± 20% ⁱ	+ 64 ± 8% ^d	+ 91 ± 12% ^j	15 ± 7% ^q	+ 74 ^j	—
	—	—	—	—	—	—
Ro15-4513	0%	+ 20%	+ 18%	53 ± 12% ^q	— 5%	—
	— 22 ± 7% ^d	— 23.3% ^d	2 ± 2% ^q	+ 56 ± 6% ^k	— 4% ⁱ	+ 70 ± 14% ^k
Zopiclone	158,	598,	1187,	—	146, ^h	—
	+ 419% ^l	+ 392% ^l	+ 344% ^l	—	+ 352% ^l	—
Zolpidem	57 ± 5.7, ^d	—	410 ± 55.4 ^d	—	—	—
	+ 133 ± 31% ^c	+ 187 ± 23% ^c	+ 142 ± 25% ^c	— 22 ± 6% ^q	— 16 ± 5% ^q	—
Zaleplon ^l	499,	1098,	1514,	—	> 3000	—
	+ 329%	+ 338%	+ 246%	—	—	—
FG8094 ^e	—	—	—	—	—	—
	— 12 ± 2%	— 12 ± 2%	— 17 ± 3%	— 3 ± 3%	—	—

FG8205 ^d	10±1.1, +54±1.4%	41±3% ^q 33±4% ^q	10±3.1, +64±5.1%	106±8%	−37±5% ^q	—
CL218872 ^d	68±28.9, +51±5.3%	—	850±456.8, +30±4.0%	—	—	—
Ocinaplon	3070, +88±8.6% ^m	3390, +52±8.8% ^m	4590, ^m +78±7.1% ⁿ	—	3790 ^m +61±4.2% ^m	—
β-CCM	—	—	—	—	—	—
β-CCt ^g	−39% ^o	−38% ^o	−32% ^o	−57% ^g	−27±2% ^q	—
DMCM	−5% 1±0.4, ^d −71±2.9% ^p	−2% —	+10% 6±4.7, ^d −62±2% ^p	—	−15% —	—
SL651,498 ⁿ	—	—	−15±5% ^q	—	−57±1% ^p	—
	~+40%	~+110%	~+80%	—	~+48% [g]	—

^aReceptors were expressed in *Xenopus* oocytes or clonal cell lines.

^bFrom [614].

^cFrom [610].

^dFrom [613].

^eFrom [617].

^fFrom [618].

^gFrom [374].

^hFrom [528].

ⁱFrom [615].

^jFrom [520].

^kFrom [607].

^lFrom [619].

^mFrom [385].

ⁿFrom [551].

^oFrom [616].

^pFrom [556].

^qFrom [583].

through the classical benzodiazepine recognition site *in vivo*. The GABA_{A1}-preferring negative modulator of GABA action β -CCt (β -carboline-3-carboxylate-*t*-butyl ester) behaviorally reverses the ataxic effects produced by zolpidem and triazolam. These data have been interpreted to indicate that antagonism at GABA_{A1} receptors is responsible for the observed behavior and that these receptors mediate ataxic effects of benzodiazepines [374].

The above pharmacological evidence could also be explained by effects of β -CCt exerted through GABA_{A1} receptors compensating for ataxic effects of zolpidem and triazolam exerted through any one of the many other GABA_A receptor subtypes these drugs interact with at pharmacologically active doses.

The α_1 -subunit KO mice develop normally and do not exhibit alterations in locomotor activity or measures of mouse anxiety levels. KO of this subunit induces and may thus be compensated for by overexpression of α_2 and α_3 subunits, which are evidently sufficient to sustain GABAergic inhibition [167, 375–377]. This deletion of α_1 is accompanied by the development of GABA-induced currents with slower decay kinetics as compared with wild-type responses in cerebellar neurons [378]. The α_1 -subunit KO mice also exhibit an increased sensitivity to the locomotor-stimulating actions of ethanol and a reduced hypnotic effect of some anesthetics [379].

In the α_1 -subunit KO mouse, diazepam continues to exhibit an effect characterized as anxiolysis using the elevated plus-maze test as a measure of mouse anxiety. These KO mice are insensitive to the anticonvulsant effect of diazepam. The hypnotic effect of a high dose of zolpidem is reduced by 66% in the KO mice as measured by the duration of the loss of the righting reflex while the effect of a high dose of diazepam is increased by 57% in these animals [375, 376].

In mice carrying the α_1 H101R mutation diazepam loses its ability to induce sedation [380–384]. In addition, the anterograde amnesic and anticonvulsant actions of diazepam are reduced in α_1 knockin mice, suggesting that these effects are mediated by α_1 -containing receptors [380, 381]. Collectively, the results in α_1 -subunit KO mice have been interpreted to indicate that this subunit is not required for the anxiolytic effects of nonselective benzodiazepines in rodents. In very recent studies, however, the role of α_1 -subunit-containing receptors in anxiolysis has been challenged as one of the compounds, ocinaplon, which have comparable affinity to α_1 - and α_2 -subunit-containing receptors displayed robust anxiolysis without sedative side effects [385]. The pharmacological effects of benzodiazepine site negative modulators or inverse agonists are opposite to those of benzodiazepine site positive modulators or agonists. However, Ro 15-4513, a benzodiazepine site inverse agonist, displays some agonistic actions in α_1 H101R knockin mice. Thus, in wild-type animals Ro 15-4513 increases locomotion and exerts proconvulsant effects whereas in α_1 H101R knockin mice this inverse agonist inhibits locomotion and is anticonvulsant. These data indicate that at least some of the pharmacological actions of benzodiazepine site inverse agonists are mediated by α_1 -subunit-containing GABA_A receptors [381].

12.13.4 α_2 Subunit

Diazepam does not act as an anxiolytic in mice carrying the α_2 H101R point mutation while it remains anxiolytic in α_1 H101R, α_3 H126R, and α_5 H105R knockin mice [383]. Importantly, this lack of response to the anxiolytic actions of benzodiazepine modulatory site ligands is specific since in α_2 H101R mice the anxiolytic effects of

sodium pentobarbital persist. Thus, it has been proposed that the anxiolytic action of diazepam is selectively mediated by α_2 -containing GABA_A receptors [383].

However, ocinaplon effectively potentiates GABA responses of α_1 -containing ($\alpha_1\beta_2\gamma_2$) receptors and is a selective anxiolytic drug with little sedative activity in humans and experimental animals, indicating that the pharmacological reality in rat, nonhuman primate, and human may be more complex than originally thought based on research in KO mice [385]. The results with ocinaplon strongly suggest that the sedative side effects of benzodiazepine positive modulators are not mediated, at least in a simple way, by potentiation of α_1 -containing receptors as suggested previously from results obtained using knockin mice [380–384].

12.13.5 α_3 Subunit

A novel 3-heteroaryl-5,6-bis(aryl)-1-methyl-2-pyridone that acts as a negative modulator (inverse agonist) selective for α_3 -containing receptors is proconvulsant and anxiogenic in rats [386]. At very high doses, diazepam loses its myorelaxant effects in the α_3 H126R knockin mice as compared with wild-type animals, suggesting that this pharmacological effect can be influenced by GABA_A receptors containing α_3 subunits [387]. The thalamic reticular nucleus that expresses only α_3 -containing GABA_A receptor regulates thalamocortical oscillations. The suppression of thalamic oscillations by clonazepam observed in slices of wild-type and α_1 H101R mice is absent in α_3 H126R mutant mice, indicating that this effect may be mediated by α_3 -containing GABA_A receptors [388].

12.13.6 α_4 Subunit

Steroid withdrawal paradigms in rats constitute models of premenstrual, postpartum, and postmenopausal syndromes and are accompanied by enhanced anxiety and susceptibility to seizures and an increase in α_4 -subunit levels. These effects are prevented by the suppression of α_4 -subunit expression, suggesting, that the upregulation of α_4 -containing GABA_A receptors leads to an increase in anxiety and neuronal excitability [389–393].

12.13.7 α_5 Subunit

The contribution of α_5 -containing receptors was studied in vivo using compound L-655,708, a partial negative allosteric modulator that exhibits at least 50-fold selectivity for α_5 -containing receptors over those containing α_1 , α_2 , α_3 , α_5 , and α_6 subunits. Elevated plus-maze performance was used to assess for the anxiolytic effects of L-655,708 (0.625–5 mg/kg i.p.) in male mice. Mice treated with L-655,708 showed a marked increase in the frequency of entries and time spent in closed arms and a decreased frequency of entries and time spent in open arms as compared with the control group. These observations suggest that L-655,708 produces an anxiogenic effect that influences elevated plus-maze performance. These observations highlight the importance of GABA_A receptors containing an α_5 subunit, which is expressed in the hippocampus, a region of the brain which has been implicated along with the amygdala in the modulation of anxiety states [394].

Two transgenic models have been generated to study the contribution of α_5 subunit. In one the entire subunit has been deleted [395], and in the second the α_5 H105R point mutation has been introduced [396]. Apart from an incomplete muscle-relaxing effect, neither the sedative, anticonvulsant, nor anxiolytic effects of diazepam were reduced in α_5 H105R mice.

Both of these genetically modified mice showed an improved performance in animal models of spatial learning and memory [395, 396], whereas performances on non-hippocampal-dependent learning and on anxiety tasks were similar to those of the wild-type control animals. These data suggest that α_5 -containing GABA_A receptors play a key role in cognitive processes by controlling a component of synaptic transmission in the CA1 region of the hippocampus and selective inhibitors of α_5 -subunit-containing receptors could have use as cognitive enhancers, for instance in mild cognitive impaired elderly or Alzheimer's disease patients [395].

12.13.8 α_6 Subunit

Studies on KO mice that lack the α_6 subunit reported no change in the response of these mice to pentobarbital, general anesthetics, or ethanol compared with wild-type mice [397], but the KO were more sensitive to the motor-impairing actions of diazepam than their wild-type counterparts [398], but not to development of tolerance to motor-impairing actions [399]. Studies of motor performance (rotarod, horizontal-wire, pole-descending, staircase, and swimming tests) reveal no differences in baseline functioning or learning among α_6 -deficient and wild-type mice. However, rotarod performance of the KO mice is significantly more impaired by diazepam (5–20 mg/kg, i.p.) when compared with wild-type mice. Ethanol (2.0–2.5 g/kg, i.p.) produces a similar impairment in α_6 -deficient and wild-type mice. Diazepam-induced ataxia in α_6 -deficient mice could be reversed by the benzodiazepine site antagonist flumazenil, indicating involvement of other GABA_A receptors. These findings suggest that GABA_A receptor α_6 -subunit-dependent actions can be compensated for by other receptor subunits, but the α_6 -subunit plays an important role in modulation of the ataxic side effects of benzodiazepines [398].

In addition, a selective posttranslational loss of the δ subunit was apparent in cerebellar granule cells, which indicates that the δ subunit is coassembled with the α_6 subunit [146]. The absence of the α_6 subunit triggered various additional changes in the cerebellum, which included a reduction in the affinity of the GABA_A receptor for muscimol [397], an increase in the number of receptors containing the β_3 subunit compared with wild type [424], and, interestingly, a compensatory upregulation of a K⁺ channel (TASK-1) in granule cells [400].

12.14 PHARMACOLOGY OF GABA_A RECEPTORS CONTAINING DIFFERENT β SUBUNITS

There are numerous observations that describe altered expression of β subunits in patients with temporal lobe epilepsy [401–403] and also in various experimental models of epilepsy [404, 405]. Deletion of the gene encoding the β_3 subunit results in mice that possess only half of the normal density of GABA_A receptors in the brain [406]. Most of these mice die in the neonatal period; however, a few survive and

grow to normal body size [407], although these mice display various neurological impairments, including hyperresponsiveness to sensory stimuli [408], strong motor impairment, and epileptic seizures [409], which might be due to the lack of β_3 -containing receptors as “desynchronizers” of neuronal activity [410, 411].

The sedative and anesthetic effects of anesthetics may also be mediated by GABA_A receptors containing different β -subunit isoforms [412–414]. Mice lacking the β_3 subunit exhibit an attenuated sedative/hypnotic response to midazolam and etomidate but respond normally to the effects of pentobarbital, enflurane, and halothane, suggesting that these agents act at different GABA_A receptor subunits [412]. Studies of sleep time comparing mice lacking the β_3 subunit with wild-type mice reveal a small but significant reduction in rapid eye movement (REM) sleep time in β_3 KO mice. Baseline electroencephalographic (EEG) delta power is significantly greater in KO mice than in wild-type mice. Although midazolam decreases non-REM sleep delta power of EEG and REM sleep time in wild-type mice, only the former response is seen in the KO mice. These results suggest that although the β_3 subunit is not the molecular site of action of midazolam, it may be involved in regulating its effects on REM sleep [415].

Studies of etomidate-insensitive mice carrying β_2 N265S mutation showed that these animals were lacking the sedative effects to subanesthetic dosages of etomidate (0.3–7.5 mg/kg). This suggests that the sedative effects produced by the etomidate are mediated by the β_2 subunit [369]. Loss of the pedal withdrawal reflex and suppression of wave amplitude in the electroencephalogram were observed in mice with an etomidate-insensitive β_2 subunit following exposure to high dosages (10–15 mg/kg) of etomidate, suggesting that the β_3 subunit is somehow involved in the modulation of anesthetic effects and loss of consciousness [369]. Furthermore, KO mice spent less time sleeping and recovered faster from the effects of etomidate than did wild-type mice, providing additional support for the conclusion that the sedative/hypnotic effects of etomidate are mediated primarily via β_2 subunits and that compounds selective for receptors containing β_3 subunits may be able to provide anesthesia with improved recovery time [369]. The latter hypothesis was confirmed in the study of knockin mice carrying the β_3 N265S mutation that rendered mice insensitive to the anesthetics propofol and etomidate. Moreover, it was observed that the potency of volatile anesthetics (enflurane and halothane) on these receptors is reduced, suggesting that it has a key role in mediating the hypnotic and immobilizing responses in vivo. However, actions of volatile anesthetics were not completely abolished and appear to involve a broader spectrum of molecular targets, for example, GABA_A receptors containing β_1 and β_2 subunits [370].

12.15 PHARMACOLOGY OF GABA_A RECEPTORS CONTAINING DIFFERENT γ SUBUNITS

In mice with a targeted disruption of the γ_2 gene diazepam fails to induce sedation and to impair the righting reflex. Furthermore, electrophysiological experiments performed in dorsal root ganglion cells indicate a lack of potentiation of GABA responses by flunitrazepam. These results are consistent with a loss of 94% of benzodiazepine binding sites in neonatal brains of γ_2 -subunit-deficient mice [416].

Most homozygous γ_2 KO mice die perinatally, and this might be due to the reduction in channel conductance that is observed in these animals and the requirement of the γ_2 subunit for synaptic clustering of GABA_A receptors [303]. Mice heterozygous for the γ_2 subunit KO mutation develop and behave normally and the synaptic clustering of GABA_A receptors is only partly reduced. These animals show a high-anxiety response to natural and learned aversive stimuli as well as a cognitive bias for threat cues [417].

In mice lacking the long splice variant of the γ_2 subunit (γ_{2L}) the sleep time after midazolam and zolpidem administration is increased compared to wild-type mice. In contrast, responses to etomidate and pentobarbital are unchanged. Although the number of GABA_A receptors was not altered in γ_{2L} KO mice, there was a corresponding increase in the affinity of brain membranes for positive modulators (midazolam, diazepam, and zolpidem), while the affinity for negative modulators (β -CCM and Ro 15-4513) was decreased. These findings suggest that absence of the long splice variant of the γ_2 subunit increases the affinity of positive modulators for GABA_A receptors that is translated into a modest increase in behavioral sensitivity to these compounds [418]. Electrophysiological studies of human GABA_A receptors expressed in *Xenopus* oocytes indicate that the modulatory effect of neurosteroids depends on the γ -subunit isoform. Receptors composed of γ_2 subunits are more sensitive to the neurosteroid 5 α -pregnan-3 α -ol-20-one compared to γ_1 -containing receptors [419].

12.16 PHARMACOLOGY OF GABA_A RECEPTORS CONTAINING δ SUBUNITS

Disruption of the gene encoding the δ subunit produces mice with a higher susceptibility to pentylenetetrazole-induced seizures [420], and this genetic deletion is accompanied by an increase in γ_2 - and a decrease in α_4 -subunit expressions [421, 422]. These KO mice display a reduction in sleep time following the administration of the neurosteroids alphaxalone and pregnanolone, whereas the response to pentobarbital, propofol, etomidate, ketamine, and midazolam is indistinguishable from that observed in wild-type mice [423].

Electrophysiological studies with recombinant GABA_A receptors expressed in heterologous expression systems indicate that the presence of δ subunits increases the sensitivity of GABA_A receptors to the stimulatory effects of neurosteroids [272, 419]. The sensitivity of GABA-evoked currents to pregnanolone sulfate, a negative allosteric modulator of GABA_A receptors, is increased in the ventral basal complex of the thalamus in δ -subunit-deficient mice. However, in the same brain region of KO mice the effect of 3 α ,5 α -tetrahydrodeoxycorticosterone, a positive allosteric modulator, is similar to wild-type mice. These data suggest that positive and negative neurosteroid modulators of GABA_A receptors have different binding sites [424]. δ -Subunit deficiency also leads to alterations in the behavioral responses to ethanol. Thus, δ KO mice present a decreased ethanol consumption, reduced withdrawal effects after chronic ethanol administration, and decreased anticonvulsant response to ethanol. However, the anxiolytic and hypothermic effects of ethanol are unaffected [425].

12.17 PHARMACOLOGY OF GABA_A RECEPTORS CONTAINING “RARE” SUBUNITS

Pharmacological properties of GABA_A receptors containing the ϵ , θ , and π subunits have not yet been studied in detail. The ϵ -subunit expression is restricted to the hypothalamus and hippocampus, suggesting a specific role regulating inhibitory neurotransmission [251]. Electrophysiological experiments using recombinant receptors indicate that expression of the ϵ subunit in combination with α and β subunits renders spontaneously active GABA_A receptor channels that are insensitive to benzodiazepines [251]. There is a disagreement about the effect of allosteric modulators on ϵ -containing GABA_A receptors. Davies et al. [426] and Belelli et al. [419] have found that substitution of the ϵ for the γ subunit abolishes the effect of neurosteroids and pentobarbital. In contrast, results from Whiting et al. [251] and Neelands et al. [162] indicate that ϵ -containing receptors are modulated by these agents.

The θ subunit shares 50% of its amino acid sequence with the β subunits. The distribution of the θ subunit is restricted to monoaminergic neurons [101].

The π subunit is expressed in the hippocampus and in nonneuronal tissues such as the uterus [94]. The amino acid sequence of this subunit is similar to that of the β (37%), δ (35%), and ρ (33%), subunits [94, 162]. It can coassemble with either $\alpha_5\beta_3$ or $\alpha_5\beta_3\gamma_3$ subunits to produce recombinant GABA_A receptors that are less sensitive to diazepam. [162]. The role of θ and π subunits in the nervous system remains to be fully explored.

12.18 STRUCTURAL DETERMINANTS OF RECEPTOR ACTIVATION

GABA is the endogenous activator of GABA_A receptors. Different compounds with distinct intrinsic activities are also recognized by the agonist binding site of the GABA_A receptor. The binding of agonists (Fig. 12.8) (e.g., GABA or muscimol) is coupled to the opening of the channel, the so-called channel gating process. Partial agonists (e.g., THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), or isoguvacine) differ from agonists with regard to channel opening efficacy. The binding of a competitive antagonist [e.g., bicuculline or gabazine (SR-95531)] stabilizes the closed state of the receptor channel (Fig. 12.9). Competitive antagonists are viewed as classic competitive inhibitors of GABA_A receptor [427], but there are indications that they can induce conformational changes [428–430].

Molecular techniques have identified residues that may participate in binding and gating of GABA channels [128, 293, 429, 431–433]. Agonist binding appears to involve multiple, discontinuous protein domains, often from separate receptor subunits [293, 419, 424, 425]. Binding can thus involve a type of chelation, or “induced fit,” process [436–439] in which separate regions of the receptor come together to interact with the agonist. A chelation mechanism implies that the agonist may reciprocally organize separate regions of the receptor into a relatively rare conformation such as an open state. Such reciprocal interactions trap agonist in the binding site [430, 440] and can be related to the conformational change produced in the agonist binding site (Fig. 12.10).

Electron diffraction measurements on nAChR have revealed the structure of this Cys loop ligand-gated channel at up to 4.6 Å resolution [221–223, 441, 442]. The conformational change in the ligand binding domain (LBD) in nAChRs produced by

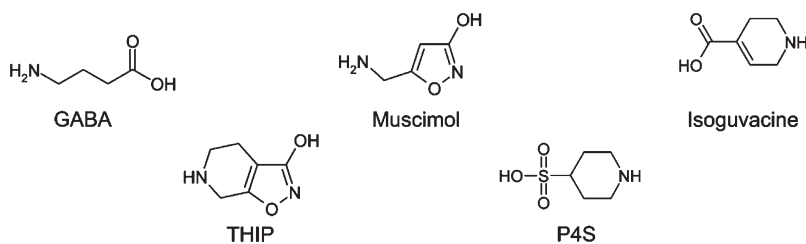


Figure 12.8 GABA_A receptor ligands: agonists (GABA, muscimol, isoguvacine) and partial agonists (THIP, P4S).

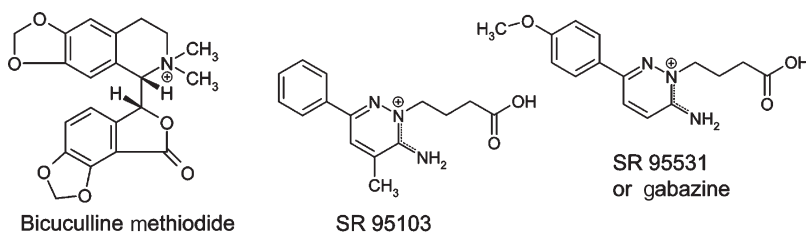


Figure 12.9 GABA_A receptor ligands: competitive antagonists.

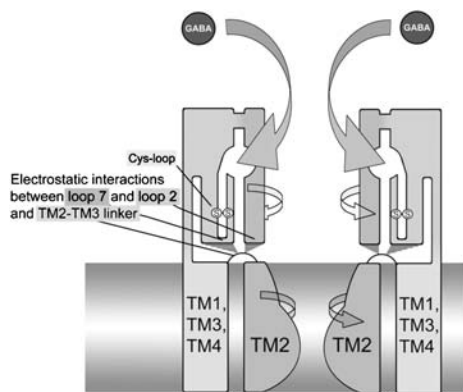


Figure 12.10 Hypothetical model of GABA_A receptor gating proposed on the basis of EM cryomicroscopy experiments performed on another Cys loop receptor—the nicotinic acetylcholine receptor [314]. The agonist-induced rotations in the extracellular domain of subunits are transmitted to the gate via TM1, which is directly linked to the receptor extracellular domain and electrostatic interaction between loop 2 and 7 and TM2–TM3 linker. The rotations destabilize the gate, causing the TM2 helices of each subunit to adopt an alternative configuration, which is permeable to the chloride ions.

agonists primarily affects the α subunits (equivalent to β subunits in GABA_A receptors), rotating their inner (pore-facing) β sheets by 15 degrees about an axis passing through the disulfide bridge and oriented perpendicular to the membrane plane [441, 442].

Agonist binding rates are often slower than expected for a diffusion-limited process [438, 443–445], implying that an energy-requiring process precedes or

accompanies binding [438]. Under the agonist chelation hypothesis, this process would correspond to structural rearrangements in the binding site that lead to channel opening. Therefore, ligands capable of opening the channel must bind slower than the diffusion limit, whereas ligands that do not open the channel (i.e., competitive antagonists) bind more rapidly than agonists [439].

12.19 ARCHITECTURE OF AGONIST BINDING SITES

Residues implicated in agonist binding are located in at least six different non contiguous extracellular N-terminal regions of the α and β subunits. These regions have been designated as loops A–F in the homologous nAChR [276, 277]. The agonist binding site of GABA_A receptors is formed by the (–) side of α and the (+) side of β subunit (Fig. 12.5a) [288, 293, 446]. Residues in different loops are likely to have different functional roles. Some residues may directly contact the agonist, some may be important for maintaining the structural integrity of the binding site, and others may mediate local conformational movements within the site. GABA binding sites composed from different α - and β -subunit isoforms may have different GABA sensitivities [447]. Two agonist molecules bind to the GABA_A receptor. Even though the two agonist sites are located at similar interfaces formed by identical (–) sides of α and (+) sides of β subunits, they have dissimilar properties: Site 2 (flanked by α and γ subunits) has higher affinity for GABA than site 1 (flanked by γ and β subunits). In contrast, muscimol and bicuculline exhibit some selectivity for site 1 [266] (Fig. 12.5).

The following residues are thought to take part in the formation of the agonist site (see Fig. 12.11): on the α_1 subunit, residues F64 [431, 448], R66, and S68 [293] on loop D; K116, R119, and I120 [449–451] on loop E; and V178, V180, and D183 [452] on loop F. Complementary residues in the β_2 subunit include Y97 and L99 [453] on loop A; Y157 and T160 [444] on loop B, and T202, S204, Y205, R207, and S209 [429, 432] on loop C. Interestingly, domains involved in the formation of the GABA and benzodiazepine binding sites are homologous (reviewed in [292]).

The spatial arrangement of these residues was unclear until direct crystallographic data of a protein involved in synaptic transmission of the snail *Lymnaea stagnalis* were obtained. This water-soluble protein is called acetylcholine binding protein (AChBP). The AChBP subunit is 210 residues long, forms a stable homopentamer [278, 455], and shares 24% sequence homology with the N-terminal part of human α_7 nicotinic receptor subunit and about 15% with subunits of GABA_A receptor family and can be used for homology modeling [456].

12.20 TRANSDUCTION OF BINDING TO GATING

To achieve gating, rearrangements induced by binding of GABA within the LBD must be transmitted to the channel gate, located within the membrane. Such conformational changes during channel activation have been detected in the regions of the receptor located near TM1, in the TM2 segment [128, 434], and in the TM2–TM3 loop [435]. The extracellular part of subunits forming the LBD is mechanically coupled with the transmembrane part at TM1, a part of the subunit where the pre-TM1 β sheet (β' 10) becomes the TM1 α helix and enters the membrane [536]. Inspection of the

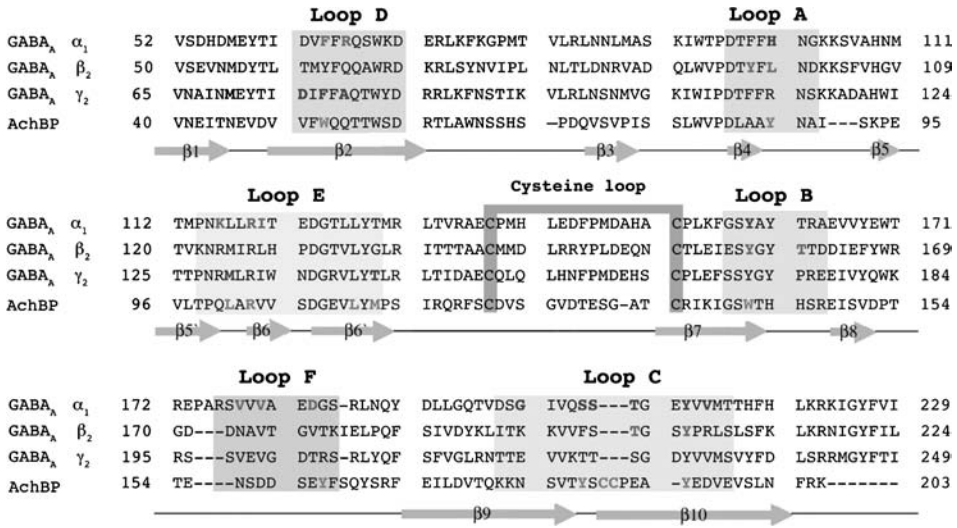


Figure 12.11 Alignment of the ligand binding domain of the GABA_A receptor α₁, β₂ and γ₂ subunits, and AChBP. Residues forming the cysteine loop are highlighted in grey. The colored boxes are six regions known in the six-loop agonist binding site model: loops A (green), B (pink), C (light brown), D (blue), E (cyan), F (light grey) [276, 277]. Residues, which are thought to form agonist binding sites, are in bold red, those implicated in the formation of benzodiazepine binding site are labeled in bold blue. Residues implicated in formation of benzodiazepine binding sites are homologous to residues forming agonist binding sites (reviewed in [294]). Residues within a radius of 5 Å of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) molecules in AChBP structure also fall within these regions, with those from the plus and minus sides of the subunit located in loops A, B and C and D, E, and F, respectively [278]. They are highlighted in bold green. Precursor peptide sequences of rat subunits were obtained from the National Center for Biotechnology Information (NCBI) (access codes: P18504 for α₁; P15432 for β₂; P22723 for γ₂). Ligand binding domains were aligned to mature peptide sequence of α₁ subunit using MaxHom [744] at Predict Protein (<http://www.predictprotein.org/>). (See color insert.)

GABA_A subunit sequences shows that TM2–TM3 loops contain conserved lysine residues, which can assist in transduction of conformational change via electrostatic interactions between charge pairs formed by loop 2 and loop 7 with the TM2–TM3 linker [457] (see Fig. 12.12).

Residues implicating loop 2 of the LBD in the transduction of conformational changes are identified on both α and β subunits in homologous positions: α₁D57 [458] and β₂D56 [459]. The distribution of residues within loop 7 of α and β subunits is non equivalent, the α₁ subunit contain α₁D149 [458] and the β₂ subunit β₂D139, β₂D146 [459], which may imply a differential role of different subunits in receptor activation. This differential role of the β₂ subunit is stressed by residue β₂K215, which is located in the pre-TM1 segment [459].

Conventional mutagenesis identified a set of residues located on the TM2–TM2 linker which upon mutation strongly affect gating; these are α₁L263 [460], α₂R274 and α₂L277 [461], α₁L276 [462], α₁K279 [458, 463], β₂K274 [459, 463], β₂L259, and γ₂L274 [460]. Charge exchange mutations and sulfhydryl trapping experiments helped to identify residues on the TM2–TM3 linker corresponding to interaction

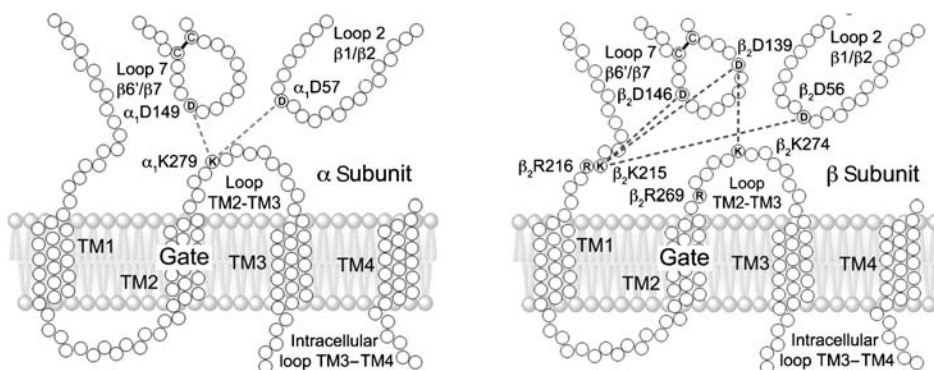


Figure 12.12 The TM2–TM3 loop is resolved in all subunits. The locations of the inner-sheet loop, loop 2 ($\beta 1/\beta 2$), and the loop 7 ($\beta 6'/\beta 7$) Cys loop disulfide bridge from the ligand binding domain are shown. In both subunits residues implicated in gating efficacy are shown as letters. In α subunit α_1 D56 in the $\beta 1/\beta 2$ loop and α_1 D149 form electrostatic interactions with α_1 K279 on TM2 and can be trapped in disulfide trapping experiment. The same is true for the β subunit, where β_2 K274 possibly interacts with β_2 D139 and pre-TM1 residue β_2 K215 interacts with β_2 D56, β_2 D139, and β_2 D146.

pairs on loop 2 and loop 7. Thus on α_1 , these interacting pairs of residues are α_1 D57 and α_1 K279 and α_1 D149 and α_1 K279. They seem to form electrostatic interactions and, as was shown in trapping experiments, they move closer to one another during gating and can be trapped with reducing agents [458]. With the aid of similar strategies, residues were found on the β_2 subunit implicated in the coupling of the LBD to the TM. These are β_2 D56, β_2 D139, and β_2 D146 that can be trapped together with residue β_2 K215 and to some lesser extent with β_2 K274 [459].

Results of Kash et al. [458, 459] suggest that there are pronounced differences in intradomain interactions of individual α and β subunits which may contribute to asymmetric propagation/distribution of conformational changes during receptor activation. The latter is supported by results obtained on homologous nAChRs during activation where binding of agonist produces 15 degrees of rotation within inner β sheets of the receptor α subunits (β in GABA_A receptors) [224, 441, 442]. Additionally, individual GABA sites display dissimilar binding properties [266]. This functional asymmetry that exists on the level of both individual receptor subunits and agonist binding sites implies asymmetrical propagation/induction of conformational changes within the receptor during activation.

The mutation of homologous residues on the γ_2 subunit (γ_2 S280 [289, 746] and γ_2 K289 [463]) located on γ_2 TM2–TM3 linker did not affect receptor gating by GABA. However, these residues located within the TM2–TM3 loop of α and γ subunits affect modulation of GABA_A receptor by benzodiazepines (see below).

12.21 THE CHANNEL PORE AND ITS ION SELECTIVITY

The channel pore is a complex symmetrical assembly of TM segments from all receptor subunits that achieves stability through productive interactions between TM segments. When the stabilizing contributions of the TM segments are perturbed sufficiently, cooperative changes can be set in motion causing the entire structure to

collapse toward an open state that allows free but selective passage of ions (Fig. 12.13).

The channel pore in all Cys loop receptors is made up from five TM2 segments, one from each subunit. Using the substituted-cysteine accessibility method 9 of 26 residues tested in the α subunit are identified as exposed to water [128]; on the β subunit the number of exposed residues is 15 of 26 tested [130].

The stability of the gate is maintained by leucine residues at the 9' turn of TM2 segment which are highly conserved among all Cys loop receptor subunits and are important for normal gating. Functional studies inferred a role for the 9' leucines (α_1 L264, β_2 L259, and γ_2 L278) in channel gating based on changes of the properties of the resting state of the receptor that produce spontaneous opening of the channel in the absence of agonist GABA [230, 460, 465–467]. Additionally, L253 was identified in β subunits and is located closer to the cytoplasmic end of the channel pore. Mutation of L253 produces receptors that are spontaneously active; that is, the open and closed states are in active equilibrium at comparable concentrations in the absence of agonist [468, 469].

The ion selectivity of all Cys loop receptors is determined by residues located at the TM1–TM2 linker; this ring of charges starts from the -1 position of TM2 according to the pore numbering system of Cys loop receptors [470–473]. Comparison of amino acid sequences of other anion channels indicates that this ring is conserved among all anion channels and formed by a proline, alanine, and arginine in the TM1–TM2 linker: α_1 P253 (β_1 P248), α_1 A254 (β_1 A249) and α_1 R255 (β_1 R250) ($-2'$ $-1'$, $0'$ respectively) [474].

The first study describing a conversion of the charge selectivity in homomeric GABA_A receptors composed of ρ subunits indicates that deletion of conserved proline at the $-2'$ turn and mutation of alanine at $-1'$ to glutamate can reverse conductance of these anionic channels [475, 476]. In heteromeric $\alpha\beta\gamma$ GABA_A receptors charge selectivity was first studied using the $\alpha_2\beta_3\gamma_2$ receptor isoform [477]. Amino acid residues in the TM1–TM2 linker of each of the three GABA_A receptor subunits were replaced by the corresponding acid residues from the nAChR

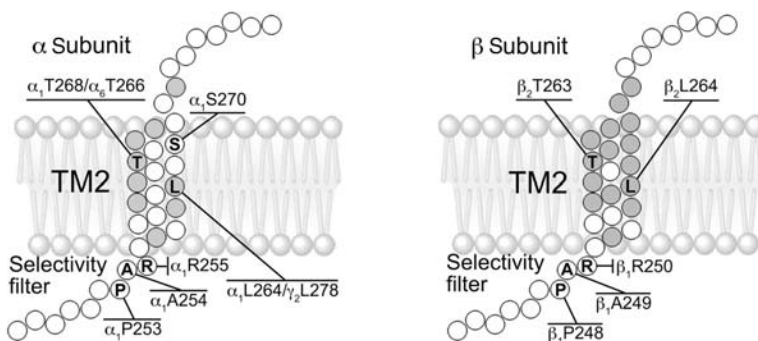


Figure 12.13 Schematic view of TM2 domain of the α and β subunits. Circles on α and β subunits filled with grey represent water-exposed residues facing the channel pore as detected by cysteine-reactive MTS (methanethiosulfonate) substances. Residues depicted on transmembrane helices are implicated in maintaining the normal properties of the gate; those shown on stretch of amino acids entering the membrane control the ion selectivity of the channel.

α_7 . Expression of a mutated β_3 subunit with wild-type α_2 and γ_2 subunits renders receptor channels that conduct cations [477]. No currents can be recorded from receptors with mutations in both the α_2 and β_3 subunits. These data indicate that the TM1–TM2 linkers of neither the α_2 nor the γ_2 subunits influence ion selectivity. Thus, ion selectivity of heteromeric $\alpha_2\beta_3\gamma_2$ GABA_A receptors is determined by β_3 subunits [477].

12.22 GABA_A RECEPTOR DESENSITIZATION AND DEACTIVATION

Virtually all ligand-gated ion channels have a set of intrinsic macroscopic properties describing their behavior in the presence of agonist; these are affinity, deactivation, and desensitization constants. Affinity is described by the sensitivity of a receptor for a given agonist. Deactivation is a characteristic delay between agonist removal and channel closing, while desensitization is the decrease in the ability of a receptor to open during the continuous presence of agonist. Desensitization is generally considered to be a negative-feedback mechanism that reduces the peak of IPSCs, but it has been suggested recently that desensitization may, in fact, enhance GABAergic transmission by prolonging IPSCs [478–480]. Jones and Westbrook [481] suggested that high-affinity, long-lived desensitized states delay unbinding of GABA from receptor, thus allowing additional late openings to occur before unbinding and slowing deactivation. Desensitization of GABA_A receptors can occur at fast (~ 10 ms), intermediate (~ 150 ms), and slow (~ 1500 ms) rates [481–484].

Additionally, receptors with specific desensitization and deactivation profiles may contribute to different types of inhibition, namely phasic and tonic. It has been observed that $\alpha\beta\gamma$ receptors containing the γ_{2L} subunit, which usually can be found at synapses, showed prominent fast desensitization accompanied by prolonged deactivation. In contrast, $\alpha\beta\delta$ receptors (with δ in place of the γ subunit) are located extrasynaptically, lack fast desensitization, and deactivate rapidly, despite having a fourfold higher affinity for GABA [484]. Thus, fast desensitization and slow deactivation might provide a mechanism that overcomes the consequences of exposing synaptic receptors to fast transients of high concentrations of neurotransmitter, whereas high-affinity, slow-desensitizing receptors are designed to sense low extrasynaptic GABA concentrations. Several studies on chimeric receptors indicate that residues located in TM1 and TM2 segments are involved in the modulation of desensitization in nACh, GABA_A, and 5-HT₃ receptors [229, 466, 485–489] (Fig. 12.14).

Studies of GABA_A receptor chimeras identify protein domains that control desensitization/deactivation kinetics. The control of desensitization and deactivation is more dependent on TM1 sequences (at least in α_1 , α_6 , γ_{2L} , δ , or ρ subunits) and less affected by the identity of TM2 segment [489–491]. Exchange mutants between the δ and γ_{2L} subunits identified two adjacent residues $\delta V233$ and $\delta Y234$ in the TM1 of the δ subunit as structural determinants of fast deactivation. Exchange of these residues to corresponding residues of the γ_{2L} subunit $\gamma_{2L}Y235$, $\gamma_{2L}F236$ confers prolonged deactivation without affecting the desensitization time course [491].

Mutation of residues α_1L264 , α_6T266 , and α_1S270 in TM2 segments, which are important for maintaining functional properties of the channel gate, increases the

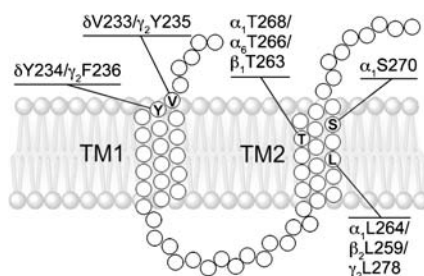


Figure 12.14 Schematic view of TM2 and TM3 of GABA_A receptor subunit. Residues shown are identified as important for specific desensitization/deactivation properties of GABA_A receptors.

gating efficacy of GABA_A receptors by slowing channel closing rates and thus prolonging deactivation [229, 492].

12.23 MODULATION OF GABA_A RECEPTOR VIA ALLOSTERIC BINDING SITES: BENZODIAZEPINE RECOGNITION SITE

In 1957, Sternbach and his colleagues at Hoffmann-La Roche discovered by serendipity that a new compound, chlordiazepoxide, decreased muscle tension, reduced fear, and was anticonvulsant in animals. A few years later diazepam was discovered [493]. These compounds were the first clinically used benzodiazepines and their discovery ushered a new era in the treatment of anxiety and related disorders [493–495].

Since the 1960s benzodiazepines have become the primary pharmacological treatment for generalized anxiety disorder, epilepsy, sleep disorders, and alcohol withdrawal and in the induction and maintenance of anesthesia [495–503]. By the mid-1970s researchers worldwide started to acknowledge a possible involvement of GABAergic systems in the action of benzodiazepines [504, 505]. Finally, by the end of the 1970s the involvement of GABA_A receptors in the action of benzodiazepines became firmly established by a number of laboratories worldwide (Figs. 12.15 and 12.11–14, 506) [11–14, 506]. The number of compounds with the benzodiazepine template has been reported to exceed 3000. Among the pharmacological agents that allosterically modulate GABA_A receptors, the benzodiazepines have gained major clinical relevance [495, 497, 498, 500].

Ligands of the benzodiazepine recognition site have been subdivided into three classes according to their intrinsic activity: positive allosteric modulators, negative allosteric modulators, and null modulators (Figs. 12.17–19). The terms “agonist,” “inverse agonist,” and “antagonist” are also used for these compounds when referring specifically to actions at the benzodiazepine recognition site. The term agonist should, however, properly be reserved for reference to GABA, which is the natural agonist at the GABA_A receptor, and the term antagonist (e.g., bicuculline) should be reserved for those compounds that block GABA agonism. Agonism and inverse agonism could properly be used when referring to the pharmacological effects of these compounds in vivo.

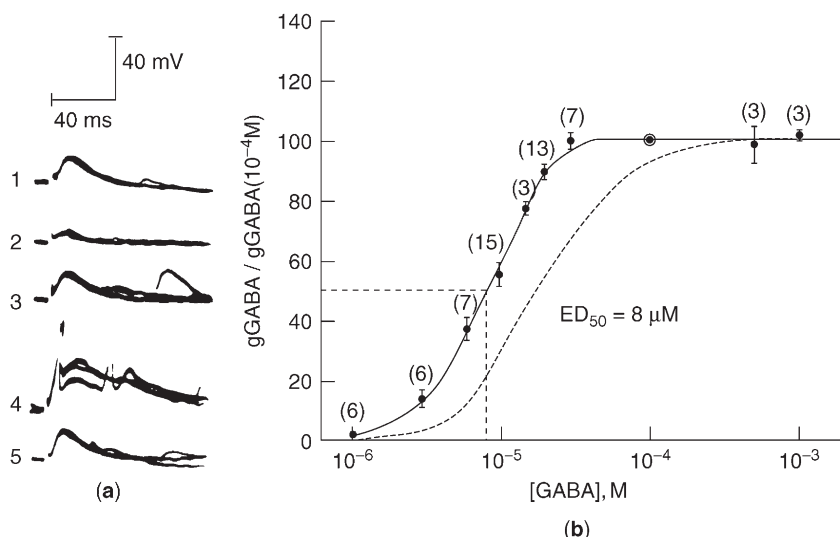


Figure 12.15 (a) Chlordiazepoxide (CDPX) potentiates stimulus-evoked, bicuculline-sensitive IPSPs. Superimposed oscilloscope traces show evoked synaptic potentials recorded with a potassium chloride electrode from the same chick spinal cord neuron. Records labeled 1–5 were obtained in sequence: 1, control; 2, during pressure injection of 100 μM bicuculline; 3, 1 min later (return to control); 4, during injection of 120 μM CDPX; 5, 2 min later. The break in each trace immediately before the evoked response is the stimulus artifact. Spontaneous synaptic potentials appear late in records. Resting potential was -80 mV. (b) Effect of CDPX on the GABA dose–response function on chick spinal cord neurons. The response to each test dose of GABA on a neuron was expressed as a fraction of the response of the same neuron to a 0.1 mM standard dose. The solid line represents the GABA response measured in the presence of 100 μM CDPX in the bath. Bars represent standard error (SE); the number of cells tested at each concentration is given in parentheses on the curve. The dashed line is a plot of the GABA dose–response function determined in the absence of CDPX (8). This curve has been normalized to the same maximum as the first curve. [These figures are reproduced from [11] (*Nature London*) and [13] (*Journal of Neurophysiology*) with permission.]

Positive allosteric modulators such as classical benzodiazepines bind to GABA_A receptors and induce a conformational change [129] increasing the affinity of binding sites for GABA without affecting the maximum GABA response. At the single-receptor level these conformational changes result in an increased probability of channel opening or gating [509]. Antagonists of the benzodiazepine site do not affect GABA-elicited responses. However, they prevent other allosteric modulators from binding and thus from allosteric modulation of receptor function. Negative allosteric modulators have opposite effects to positive allosteric modulators, decreasing affinity for GABA.

The use of classical benzodiazepines is often associated with side effects. The sedative effects associated with benzodiazepines can impair an individual to operate a machinery and thus those individuals whose livelihood depends on their ability to operate heavy equipment such as a bulldozer or forklift may not be willing or able to take the medication while at work without jeopardizing their job.

Sedation is not the only side effect: Daytime drowsiness with long-acting compounds which may even accumulate in the body with multiple use or rebound

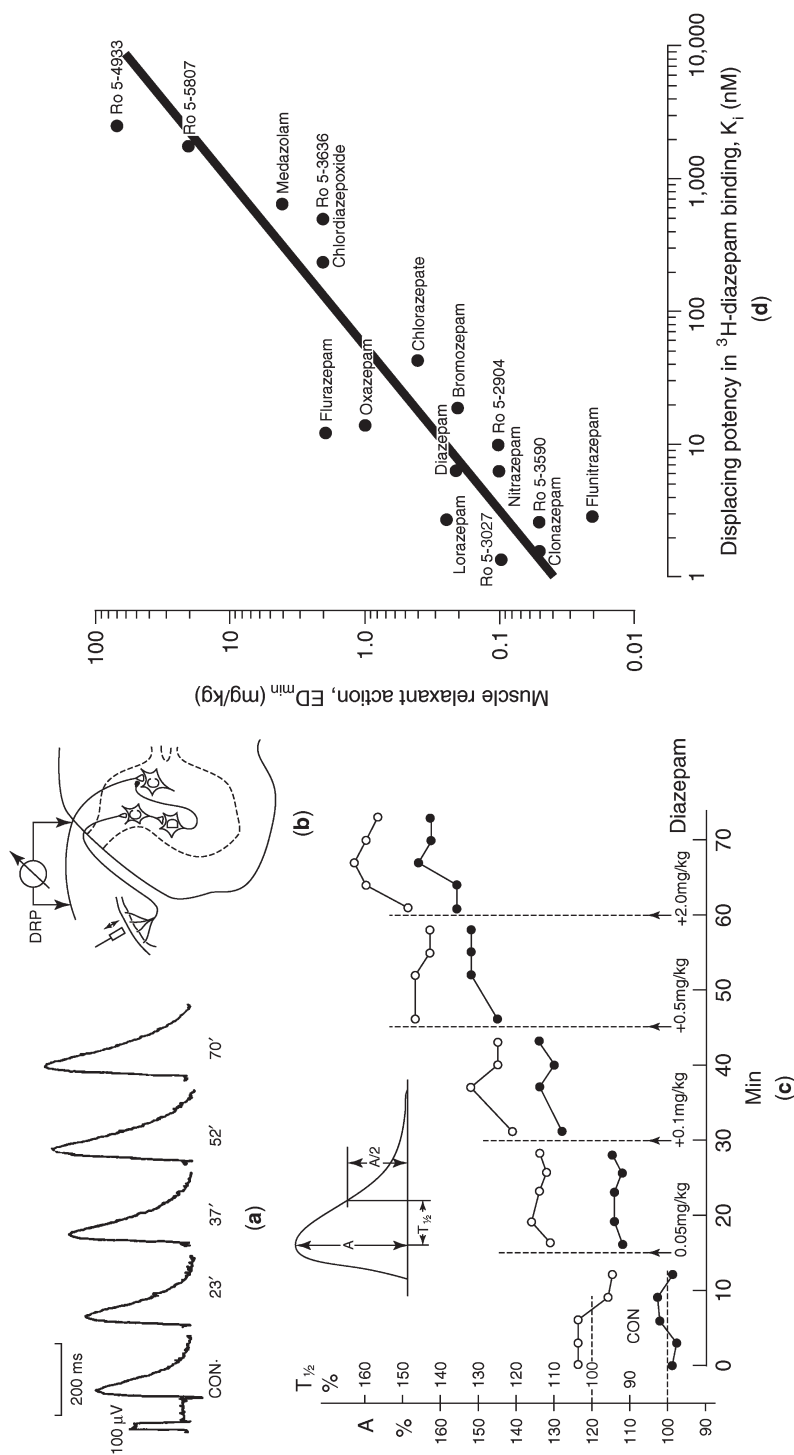


Figure 12.16 First demonstration of an enhancement of presynaptic inhibition in the vertebrate central system by a benzodiazepine. (a) The intensity of dorsal root potentials before (CON) and after four successive applications of diazepam following mechanical stimulation of the central foot pad. (b) Neuronal arrangement for stimulation of the foot pad and recording of the dorsal root potentials (DRPs). (c) The left-hand y axis shows the effect of successive applications of several concentrations of diazepam on the amplitude of the DRPs (A , filled circles). The right-hand y axis indicates the effect of diazepam on the time to half decay of the dorsal root potentials ($T_{1/2}$, open circles). (Reproduced from Schmidt [507], with permission of *Ergebnisse der Physiologie*.) (d) Correlation between K_i values for the inhibition of specific $[^3\text{H}]$ diazepam binding by various benzodiazepines and their pharmacological potency (cat muscle relaxant action, minimum effective dose (ED_{min}) in milligrams per kilogram, orally); correlation coefficient $r = 0.905$ ($p < 0.001$). (Reproduced from Mohler and Okada, [508] with permission of *Science*.)

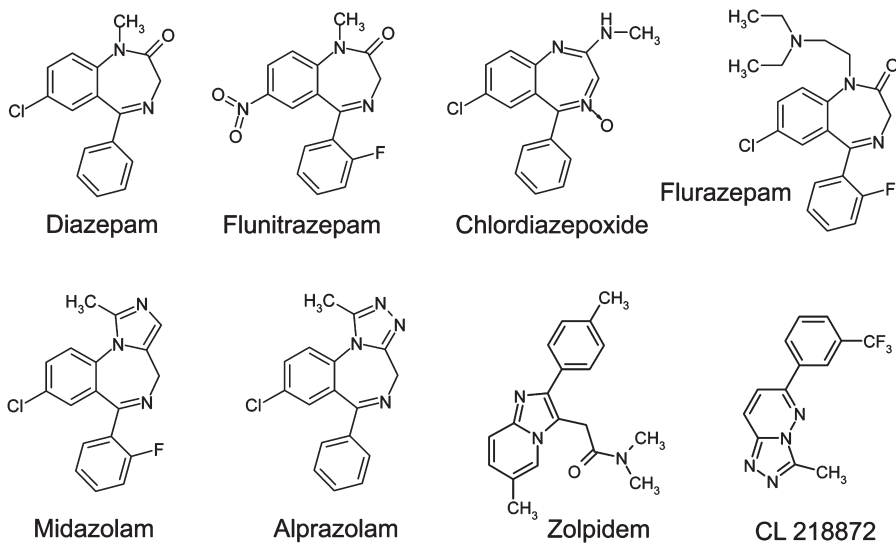


Figure 12.17 Selected positive allosteric modulators acting at benzodiazepine binding site.

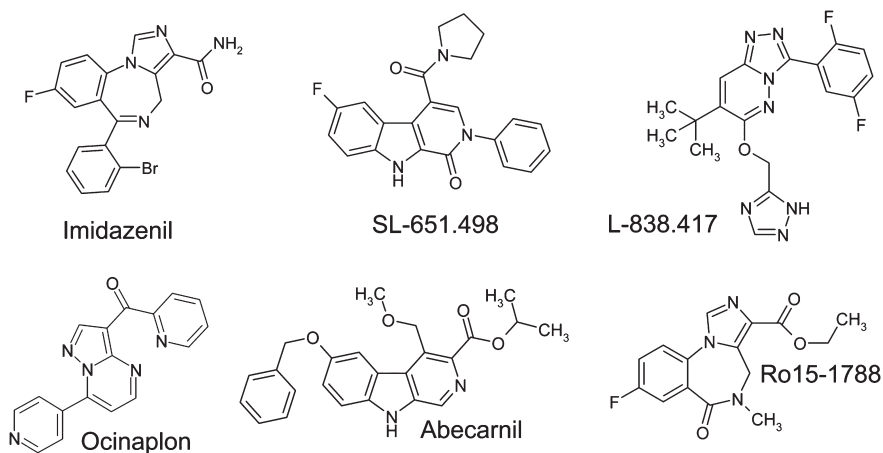


Figure 12.18 Some selected partial positive allosteric modulators and an antagonist (Ro15-1788) acting at the benzodiazepine binding site.

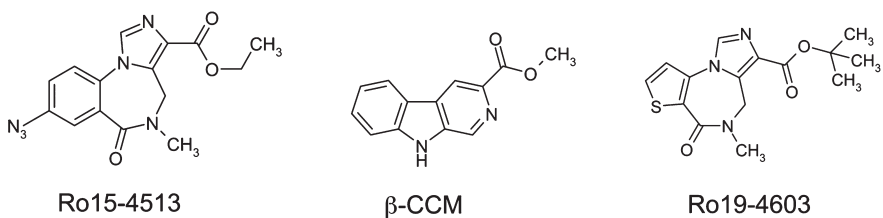


Figure 12.19 Some selected partial (Ro15-4513) and full negative allosteric modulators (β-CCM and Ro19-4603) acting at the benzodiazepine binding site.

after the use of short-acting drugs and development of tolerance and dependence also limit the current use of these drugs in the treatment of certain types of chronic anxiety disorders such as generalized anxiety disorder, epilepsy, and insomnia to the short-term medication [499, 510–512]. Anterograde amnesia, psychotic states, and alterations in sleep architecture have also been related to the use of some short-acting benzodiazepines. This has been the impetus for research into the development of effective anxiolytic compounds that do not have the side effects of the classical benzodiazepines [513, 514].

Development of such novel medicines devoid of side effects can be approached by creating compounds which display profound functional selectivity [594] or by targeting specific GABA_A receptor subtypes with subtype-selective drugs which will be expected to display fewer side effects, such as tolerance and dependence liability, because they will affect only a small population of GABA_A receptors [499–503]. In an attempt to create drugs with the desired therapeutic effects several compounds such as abecarnil [515–523], bretazenil [520, 524–530], divaplon [531–533], imidazenil [519, 534–538], zaleplon [539, 540], zolpidem [541, 542], and ocinaplon [385] have been synthesized.

Intrinsic activities of these compounds at different GABA_A receptor subtypes vary from partial to full positive allosteric modulators. For instance, abecarnil is a partial modulator at α_5 and a full positive modulator at α_1 - and α_3 -containing GABA_A receptors [600]. On the other hand, divaplon is a partial agonist at both α_5 - and α_3 -containing GABA_A receptors [520], and bretazenil and imidazenil both have low efficacy at a broad spectrum of GABA receptor subtypes [535]. These compounds exhibit fewer side effects associated with conventional anxiolytic benzodiazepines. A possible explanation for this diminished side-effect profile may be that the lower intrinsic efficacy of the GABA_A receptor partial agonists is probably sufficient to maintain low-efficacy responses such as anxiolytic and anticonvulsant actions but insufficient to induce sedation, myorelaxation, dependence, and ethanol potentiation, all of which may require high fractional receptor occupancy [543–546].

Accordingly, abecarnil is a potent anxiolytic and elicits little or weak effects in tests of motor incoordination and muscle relaxation and (in contrast to diazepam) exhibits a relatively weak ability to potentiate the effects of ethanol and barbiturates [516]. Interestingly, abecarnil was also found to be most effective with the fewest side effects at the lowest dosage studied in a double-blind, placebo-controlled clinical trial for treatment of generalized anxiety disorder [513]. The therapeutic safety, defined as the ratio between the anticonvulsant efficacy and the muscle relaxant action, is more favorable for abecarnil as compared to diazepam in rodents and baboon *Papio papio* [515]. Abecarnil also elicits potent anticonflict and taming effects with little sedative and ataxic effects in primates [521]. Chronic administration of abecarnil was found to elicit persistent anxiolytic and anticonvulsant effects without any amnesia in rats [522], and it did not induce tolerance or withdrawal syndromes in mice [518]. However, despite promising data obtained in animal models abecarnil failed to produce selective anxiolysis during clinical trials in humans [523].

The imidazobenzodiazepinone bretazenil (Ro 16-6028) acts as a partial allosteric modulator of benzodiazepine receptors producing only a 25% potentiation even at 100% receptor occupancy. These in vitro observations can be related to in vivo responses in animal models (mice) which indicate that bretazenil does not protect against seizures as well as the full allosteric positive modulator diazepam [495]. By

contrast, studies in humans and animals indicate that bretazenil elicits potent anticonflict and anticonvulsant activity and can provide these effects with a lower degree of sedation [524], dependence liability [527, 530], ethanol potentiation [535], motor impairments, and tolerance as compared to conventional benzodiazepines [544, 546–548]. Although bretazenil has lower abuse liability than diazepam, its development was apparently discontinued [529]. Likewise, divaplon elicits potent anticonflict activity and produces lower sedation [531] and tolerance [532, 533] as compared to diazepam.

Imidazenil is characterized as a partial allosteric modulator of GABA_A receptors. Behavioral experiments in the rat, using the Vogel conflict–punishment test, suggest that imidazenil is an anxiolytic. Imidazenil is devoid of sedation, ataxia, and ethanol potentiation and blocks the sedative and ataxic effects of diazepam in rats [534]. Furthermore, when administered with diazepam, imidazenil dose dependently blocks the side effects of sedation and ataxia typically associated with diazepam and thus, like flumazenil, appears to act as an antagonist on these unwanted responses [534]. It also completely attenuates the benzodiazepine-induced cognition deficit in monkeys [538]. Furthermore, it causes only low tolerance and dependence liabilities in rats [535]. Chronic treatment with a pharmacologically effective dose of imidazenil failed to induce tolerance to the effects of this drug on GABA_A receptor function in mouse brain [536].

Among the clinically used benzodiazepine site ligands only the hypnotics zaleplon and zolpidem have a pronounced preferential subtype selectivity [539–542] (Tables 12.1 and 12.2). Zaleplon was the first GABA_A receptor subtype preferring drug developed for the treatment of insomnia [539, 540]. Its binding affinity at α_1 - and α_3 -containing receptors is 8- to 20-fold higher than for receptors containing α_2 , α_4 , and α_5 subunits [549]. It has been suggested that such selectivity makes zaleplon a potent hypnotic with diminished rebound side effects.

Recent insights into the subtype specificity of benzodiazepine actions have provided guidelines for the development of subtype-selective benzodiazepine site ligands. Some of these novel developments are under experimental or clinical investigation [502, 550] and will be discussed below.

SL65.1498 (6-fluoro-9-methyl-2-phenyl-4-(pyrrolidin-1-ylcarbonyl)-2,9-dihydro-1*H*-pyrido[3,4-*b*]indol-1-one) is a pyridoindole that shows higher affinity for GABA_A receptors composed of α_1 , α_2 , and α_3 compared with α_5 . In addition, it is a full positive allosteric modulator at α_2 - and α_3 -containing receptors, whereas it acts as a partial positive modulator at α_1 - and α_5 -subunit-containing GABA_A receptors [551, 552]. Its selectivity for α_2 - and α_3 -containing GABA_A receptors may be responsible for the potent anxiolytic action in animal models (punished lever pressing, punished drinking, elevated plus maze, light/dark test) without impaired motor coordination (e.g., rotarod) or working memory (Morris water maze) [553].

Behavioral studies performed with α -subunit knockin mice suggest that ligands selective for GABA_A receptors containing α_2 subunits will be anxiolytic and will lack the sedative component [463]. However, ocinaplon, a pyrazolo[1,5-*a*]-pyrimidine, is an anxioreactive compound, which fails to show a selectivity for receptors composed of α_2 subunits. In fact, this compound, which has been shown to be an effective anxiolytic in preclinical and clinical tests, exhibits certain selectivity for α_1 -subunit-containing GABA_A receptors [385].

Discovery of subtype-specific positive or negative modulators of GABA_A receptors might be useful for the treatment of neuropsychiatric disorders extending

beyond the spectrum of pharmacological properties exhibited by classical benzodiazepines. In pursuit of a drug to enhance cognition, a series of novel ligands selective for the GABA_A receptors containing α_5 subunits have been developed. Some of these compounds have higher binding affinity for the GABA_A receptor containing the α_5 -subunit subtype compared to α_1 -, α_2 -, and α_3 -subunit-containing receptors (e.g., L-655,708) [554a]. Several compounds exhibit functional α_5 subunit selectivity as full/partial positive allosteric modulators [384], null modulators (antagonists) [554], or negative allosteric modulators [555, 556]. Such negative allosteric modulators of receptors containing α_5 subunits enhance cognitive performance in rats in the delayed matching-to-place Morris water maze test—a hippocampal-dependent memory task—without the convulsant or proconvulsant activity associated with nonselective GABA_A receptor inverse agonists [555, 556].

The novel benzodiazepine site ligand L-838,417 has comparable binding affinities toward GABA_A receptors containing α_1 , α_2 , and α_3 subunits but exhibits a threefold lower affinity toward receptors containing the α_5 subunit. L-838,417 is a null modulator at α_1 but acts as a full/partial modulator at α_2 -, α_3 -, and α_5 -containing receptors [384]. This compound has a high potency in anxiolytic (elevated plus maze and fear-potentiated startle) and anticonvulsant tests (pentylenetetrazole, audiogenic seizures) at doses that occupy less than 50% of benzodiazepine sites. Furthermore, at concentrations that occupy ~95% of benzodiazepine sites, L-838,417 failed to impair motor performance (rotarod test, chain-pulling test) [384].

Drug screening from traditional medicinal herbs has also attracted much attention in the hope of identifying novel therapeutics for the treatment of anxiety and related diseases [557]. The discovery of chrysin, one of the first flavonoids isolated from the plant *Passiflora coerulea* that interacts with the benzodiazepine binding site, marked the search for such natural anxiolytics [558–560]. A number of other naturally occurring flavonoids such as K36 [561], hispidulin [562], wogonin [563, 564], and related compounds [565–568] have been found to possess allosteric modulatory action at the GABA_A receptor complex.

K36 elicited significant behavioral responses in the elevated plus-maze test, increasing entries to the open arms (taken as an indicator of anxiolysis), with no change in locomotor activity. Moreover, in the hole-board, horizontal-wire, and rotarod tests K36 did not elicit significant sedation, myorelaxation, and motor incoordination at the anxiolytic dosages [561].

Hispidulin at concentrations of 50 nM and higher stimulated GABA-induced chloride currents acts as a positive allosteric modulator of the $\alpha_X\beta_2\gamma_{2S}$ ($X = 1, 2, 3, 5, 6$) GABA_A receptor subtypes. In contrast to diazepam, hispidulin modulated the $\alpha_6\beta_2\gamma_{2S}$ GABA_A receptor subtype. When fed to seizure-prone Mongolian gerbils (*Meriones unguiculatus*) in a model of epilepsy, hispidulin [10 mg/kg body weight (BW) per day] and diazepam (2 mg/kg BW per day) markedly reduced the number of animals suffering from seizures to 30 and 25% of animals in the respective treatment groups, versus 80% in the vehicle group after seven days of treatment [562]. Mice that received oral wogonin (7.5–30 mg/kg) exhibited a reduction in measures characteristic of anxiolytic action and similar to those elicited by diazepam in the elevated plus-maze test while showing no sign of sedation [564].

Naturally occurring flavonoids often bind to the benzodiazepine binding site with only moderate affinities. However, through synthesis of chemical libraries and molecular modeling of the flavonoid binding to the pharmacophore of the benzodia-

zepine binding site [569–572], several groups have been able to generate synthetic derivatives with higher affinities with allosteric properties varying from positive allosteric modulators to compounds devoid of intrinsic activity (antagonists) [573–576].

12.24 BENZODIAZEPINE BINDING SITE OF GABA_A RECEPTORS

Present evidence suggests that GABA_A receptors are the only effector sites of benzodiazepines in the CNS. An additional recognition site for benzodiazepine binding is located in peripheral tissue, but this is structurally and functionally unrelated to GABA_A receptors. The so-called peripheral benzodiazepine receptor is located on an 18-kDa protein in the inner mitochondrial membrane (for review see [577]). This review will focus on the recognition sites located in nervous tissue.

The nervous tissue-specific benzodiazepine (BZ) binding site has been shown to be located at the interface between the α and γ subunits, with residues from each subunit contributing to the binding site [294, 578–583] (Fig. 12.5). Photoaffinity labeling of the receptor by the benzodiazepines [³H]flunitrazepam and [³H]Ro15-4513 has been performed in order to identify residues that are part of the binding site [584–591]. The residues H101 (rat numbering) [588, 591–593] and P97 [591] have been shown to be the major sites of incorporation of [³H]flunitrazepam into the α_1 subunits. The amino acid(s) photolabeled by [³H]Ro15-4513 are contained within a subunit fragment extending from residue 104 to the C-terminus of the α_1 subunit [592], possibly within amino acids 247–289 spanning the end of TM1 to the beginning of TM3 [589]. The results from a recent study suggest that [³H]Ro15-4513 is photoincorporated into α_1 Y209 and in homologous positions in the α_2 and α_3 subunits [590].

Extensive mutagenesis experiments have also identified other α_1 residues implicated in benzodiazepine binding. The significance of α_1 H101 was initially demonstrated by substituting arginine, the native residue at the homologous position in α_4 and α_6 subunits [594, 595, 751, 752]. Substitution of histidine-101 by arginine resulted in a 500–800-fold decrease in the binding affinity of classical benzodiazepines [594, 595, 751, 752]. Experiments with receptor subunits in which residues suspected of facing the interior of the binding pocket were mutated to cysteine identified the position of classical BZ ligand (diazepam) binding relative to α_1 H101 by using BZ ligands carrying cysteine-reactive groups to link to mutated receptor subunits. The results point to interactions with the C-7 position of diazepam. Interestingly, at the functional level, the reacted receptor was irreversibly stabilized in an allosterically stimulated state [754].

The following residues in the α_1 subunit were shown to alter either benzodiazepine sensitivity in functional assays or benzodiazepine affinity in binding studies: Y159 [454], Y209 [291, 454], T162 [595], G200 [596–598], T206 [454], V211 [599], and I215 [600]. In the γ_2 subunit M57 [292] and Y59 [601] were found to be essential determinants for conferring high affinity for classical and atypical benzodiazepines. The γ_2 F77 residue is absolutely crucial for recognition of classical benzodiazepine ligands [290, 602, 603]. It should be noticed that this residue is homologous to F64 in the α_1 subunit, which has been previously shown to be a key determinant of the GABA binding site, suggesting conservative motifs between different ligand binding sites on the GABA_A receptor [290, 431, 448, 603] (Fig. 12.11). By analogy to the GABA binding pocket the residues A79 and T81 which are clustered on a β strand

around F77 are found to line up part of binding pocket [288, 601]. The residue γ_2 M130 is required for high-affinity binding of benzodiazepine binding site ligands [292, 602, 603]. Finally, a threonine residue at position 142 of the γ_2 subunit is implicated in the efficacy of benzodiazepine binding site ligands [604].

Residues identified in the BZ binding site can be divided into two groups: The first group affects only binding and the second both binding and allosteric modulation. Among those residues affecting allosteric modulation are α_1 H101, γ_2 77, and γ_2 T142. Receptors containing α subunits carrying point mutations in residues homologous to α_1 H101 exhibit lower affinity and altered efficacy for various BZ site ligands: changing partial allosteric modulators into full positive modulators (bretazenil) [752], null modulators into positive modulators (Ro15-1788) [751, 753], and partial negative modulators into positive modulators (Ro15-4513) [751, 752], thus demonstrating the importance of α_1 H101 to allosteric modulation.

In the γ_2 subunit, mutagenesis experiments have identified two amino acids, γ_2 F77 and γ_2 T142, that may play a role in the efficacy of BZ ligands. The γ_2 T142S mutation alters the efficacy of several BZ ligands; both the null modulator (Ro15-1788) and a partial negative modulator (Ro15-4513) were converted to partial positive modulators [604]. The γ_2 F77L [290, 757], γ_2 F77I [290], and γ_2 F77W [290], mutations enhanced diazepam positive allosteric modulation, even though the binding affinity of diazepam was reduced.

12.25 STRUCTURAL DETERMINANTS OF ALLOSTERIC MODULATION BY BENZODIAZEPINES

The binding of benzodiazepines changes the properties of the GABA_A receptor, but what is changed and how does the change occur? Historically, benzodiazepines were thought to act by binding to the benzodiazepine binding site and causing a change in receptor conformation via allosterism. In the case of positive allosteric modulators, the apparent affinity of the neurotransmitter recognition site for the agonist, GABA, increases, and negative allosteric modulators decrease receptor affinity for GABA [15, 294, 427]. Although this simple hypothesis has been in use since 1977, it was not until the late 1990s that data underlying both the molecular and mechanistic aspects of allosteric modulation started to emerge.

Allosteric modulation is a complex phenomenon, but as in the case of activation of ligand-gated ion channels by agonists, a multistep mechanism following binding of modulators is easily envisaged. The first step is chelation during which the ligand, upon binding, coordinates separate regions of the BZ binding site into a relatively rare conformation that causes the second step, the triggering of a conformational change in the whole receptor protein, that alter either one or both agonist binding site(s).

Rogers et al. [747] proposed an allosteric model in which diazepam increases the rate of entry into the singly bound closed state, although it was not known whether the increased rate occurs at one or both GABA recognition sites. In 1996, Lavoie and Twyman proposed an allosteric model in which diazepam accelerates the rate of GABA association at one of the sites, but the identity of each site was not specified [748]. Williams and Akabas [129], using molecular biology techniques combined with electrophysiology, subsequently determined that when certain amino acids in TM3

of the α subunit are mutated to cysteine a cysteine-reactive agent modifies two of these residues when the receptor is exposed to GABA and diazepam. The results suggest that diazepam binding changes α -subunit conformation such that only one mutated residue is accessible to modification, whereas when the GABA binds, the second or both residues are accessible.

Baur and Sigel [464] used receptors formed by concatenated subunits in which it is possible to destroy selectively one of the two agonist sites introducing point mutation. They found that both receptors with either site 1 or site 2 destroyed could be modulated by diazepam. This argues against the hypothesis that only one agonist site is altered by the binding the ligand to the benzodiazepine recognition site and suggests that modulation is not limited exclusively to one of the two agonist binding sites.

But what are the structural elements involved in transduction of allosteric modulation of GABA_A-R induced by classical BZs and how the mechanism of allosteric modulation is conceived given the fact that there are negative, null, and positive allosteric modulators? One study showed that α/γ chimeric receptors carrying a stretch of 167 aminoacids from N-terminal domain of the γ_{2S} subunit are able to bind BZ site ligands [749]. Although certain chimeric receptors exhibit wild-type binding properties and retain sensitivity to negative allosteric modulators, positive allosteric modulators display reduced efficacy in functional experiments. The stretch of amino acids from P161 to L187 on the α_6 subunit contains a region responsible for the functional positive allosteric action of Ro15-1788 on $\alpha_6\beta_2\gamma_2$ receptors [753]. However, the results of both studies agree that additional domains are important for conversion of binding into allosteric modulation.

Perhaps not too surprisingly, domains implicated in the transduction of agonist binding to channel gating in the α and β subunits are playing the very same role in transduction of allosteric modulation in the γ subunit. There is a body of evidence implying that the same parts of GABA_A receptors that are involved in the transduction of agonist binding to gating are also important for allosteric modulation—the TM1 and TM2 domains and the TM2–TM3 linker of the γ_{2S} subunit.

Studies using chimeric receptors and amino acid replacement at key residues in subunits demonstrate that the following two groups of residues were involved in transduction of conformational change induced by binding of BZ site ligands into modulation of receptor function: residues γ_2Y235 , γ_2F236 , γ_2T237 in TM1 [746]. In the TM2 segment, the part of the receptor forming the channel pore, residues γ_2S280 , γ_2T281 , and γ_2I282 were implicated in the disruption of allosteric modulation [746]. On the TM2–TM3 loop itself only one residue implicated in allosteric modulation by BZ ligands— γ_2S291 , has been so far identified [289].

Thus, allosteric coupling between the benzodiazepine and agonist recognition sites requires residues not only in the LBD [749] but also in the TM1 and TM2 domains [746] as well as the TM2–TM3 linker of the γ subunits [289, 463, 746]. When taken together the data suggest an alternative hypothesis for BZ modulation whereby binding of a benzodiazepine recognition site ligand results in a conformational change in the γ subunit that is transferred to the γ subunit TM2 domain via TM1 and the TM2–TM3 linker region. Destabilization of the γ_2 -subunit TM2 domain can in turn perturb the stability of TM2 in the α and β subunits that can lead to channel opening. This was observed by Downing et al. [758] in experiments with $\alpha_1L263S\beta_2\gamma_2$ receptors using diazepam and flurazepam; interestingly, openings gated by

flurazepam were inhibited by the BZ null modulator (antagonist) Ro15-1788. Thus, conformational instability induced in TM2 segments of other subunits can either shift the equilibrium toward the open state or be transferred back to the LBD through TM1, loops 2 and 7, and the TM2–TM3 linker, changing the conformation of the α/β -subunit interfaces that harbor agonist sites, subsequently affecting the kinetics of GABA binding to both agonist binding sites [289, 464]. The role that is played by residues located within the LBD in transduction of conformational changes remains to be determined. Additionally, the role, if any, of the low-affinity benzodiazepine site [750], which is also located in the TM region and involved in allosteric modulation of GABA_A receptors by ligands of the BZ site at high concentrations [750, 755, 756], remains to be established.

12.26 BENZODIAZEPINE BINDING SITES COMPOSED OF DIFFERENT α - AND γ -SUBUNIT ISOFORMS

Different α - and γ -subunit isoforms can assemble to form benzodiazepine recognition sites with different pharmacological properties. GABA receptors have been classified pharmacologically into diazepam-sensitive receptors that recognize classical, 5-phenyl-1,4-benzodiazepines (e.g., diazepam and flunitrazepam) and diazepam-insensitive receptors that do not recognize these ligands (Fig. 12.20) [605–607].

Diazepam-sensitive receptors can be further subdivided by their affinity to a triazolopyridazine, CL-218,872. The α_1 -containing receptors have a high affinity for CL-218,872 and are referred to as type I benzodiazepine receptors. Receptors composed of α_2 , α_3 , or α_5 subunits have a low affinity for CL-218,872 and are referred to as type II benzodiazepine receptors [608, 609]. Data showing the influence of the α -subunit isoform on the receptor pharmacology are summarized in Tables 12.1 and 12.2.

The term *benzodiazepine receptor* derives from the era in which ligand binding and electrophysiology were carried out on tissue preparations and neurons but it was not

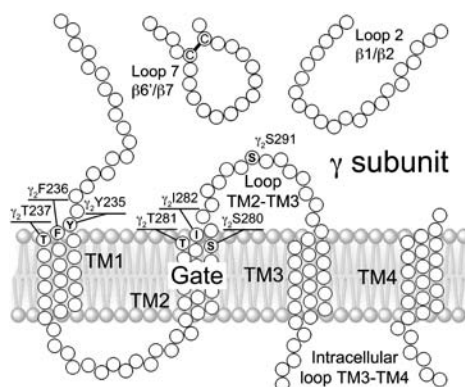


Figure 12.20 Schematic view of N-terminal and TM domain of the γ subunit. Residues located in TM1, TM2, and TM2–TM3 linker that are implicated in the transduction of allosteric modulation induced by benzodiazepine site ligands are shown in the circles. The grey circle represents part of receptor discovered in studies of chimeric subunits by Boileau et al [749].

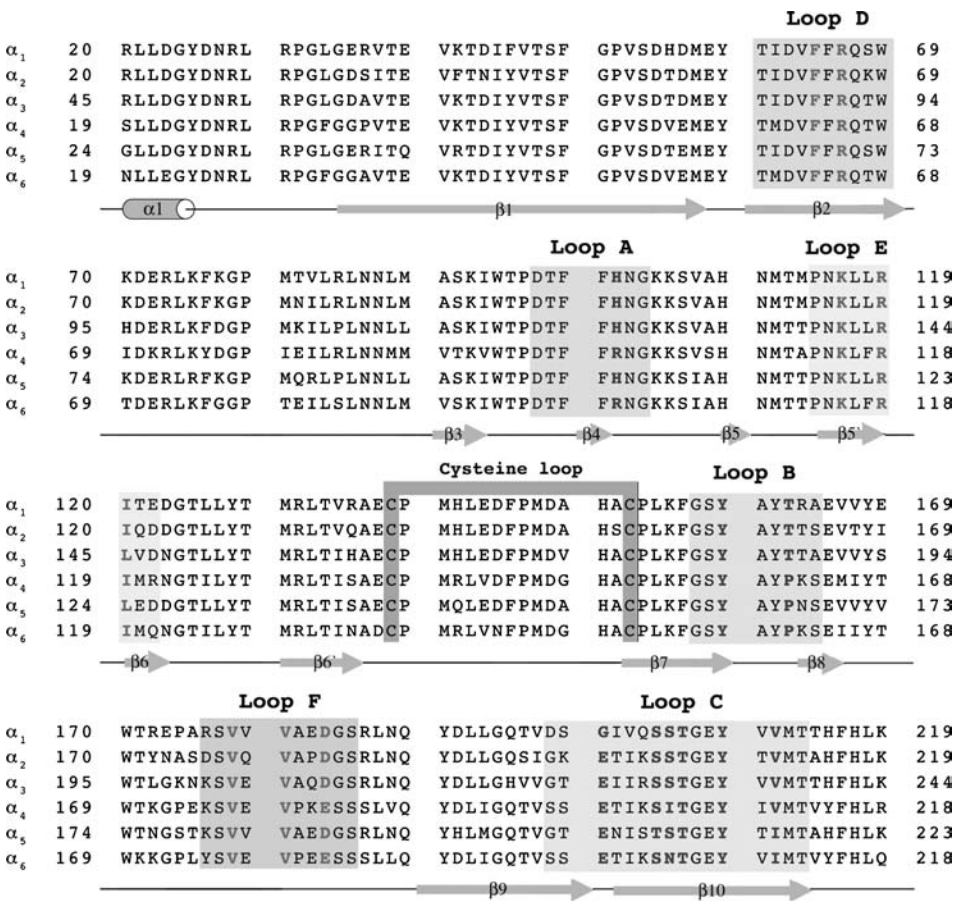


Figure 12.21 Alignment of the ligand binding domains of the GABA_A receptor α subunits. Residues forming the cysteine loop are highlighted in grey. The colored boxes are six regions, known in the six-loop agonist binding site model: loops A (green), B (pink), C (light brown), D (blue), E (cyan), F (dark green). Residues thought to form agonist binding site are in bold red, those implicated in the formation of the benzodiazepine binding site are in bold blue. Precursor peptide sequences of human subunits were obtained from NCBI (access codes: α_1 : P14867; α_2 : P47869; α_3 : P34903; α_4 : P48169; α_5 : P31644; α_6 : Q16445). Ligand binding domains were aligned to the mature peptide sequence of the α subunit using MaxHom [744] at Predict Protein (<http://www.predictprotein.org/>). (See color insert.)

known conclusively whether the functionally coupled benzodiazepine recognition site was on or associated with the type A GABA receptor. Evidence for a physical linkage between the two became possible once it was determined that multiple subtypes of the receptor existed and the subtypes were cloned. Mutagenesis experiments described in this review have demonstrated that both the GABA and benzodiazepine recognition sites are located on the GABA receptor. Whereas usage of the term benzodiazepine receptor is no longer justified, *benzodiazepine recognition site agonist*, *antagonist*, and *inverse agonist* persist as a shorthand notation when discussion focuses specifically on the benzodiazepine recognition site.

The relative preference of CL-218,872 and zolpidem toward α_1 -containing receptors (as compared with other α subunits) is conferred mainly by α_1 T161 [595, 623], α_1 G201 [596, 597], α_1 S205 [595, 623], and α_1 V211 [599]. It is noticeable that the subunits conferring the higher affinity to zolpidem have the smaller amino acid residues at both positions 201 and 211, suggesting a steric role for these residues in benzodiazepine selectivity. It was proposed that a similar mechanism also underlies more than 1000-fold decrease in affinity for diazepam, flunitrazepam, zolpidem, and CL-218,872 at α_6 -containing receptors compared to those having the α_1 subunit [598, 599, 606, 625]. α_6 N204 is another residue conferring insensitivity of α_6 -subunit-containing receptors to β -carboline, β -CCE. Introduction of a point mutation α_6 N204S or α_6 N204I found in the homologous position of α_1 or α_4 subunits restores affinity [625].

Many studies [586, 587, 616, 617, 626] indicate that the γ subunit also contributes significantly to the properties of the benzodiazepine recognition site (Fig. 12.21). GABA_A receptors containing γ_1 or γ_3 subunits have a lower affinity for most of the classical benzodiazepines compared to receptors composed of γ_2 subunits. In addition, γ_1 -containing receptors have a lower affinity for the antagonist Ro15-1788 than those receptors containing γ_2 or γ_3 subunits [150, 602, 615]. Data showing the influence of the γ -subunit isoform on GABA_A receptor pharmacology are summarized in Tables 12.3 and 12.4.

Two amino acid residues are determining the selectivity of zolpidem for γ_2 -subunit-containing receptors [290, 292, 602]. These are γ_2 F77 and γ_2 M130; the γ_1 subunit has an isoleucine (γ_1 I79) at a position homologous to γ_2 F77 and additionally γ_1 and γ_3 subunits contain a leucine at a position homologous to γ_2 M130 (γ_1 L132 and γ_3 L133). Introduction of the point mutations γ_1 I79F and γ_1 L132M in the γ_1 subunit and γ_3 L133M in γ_3 restores zolpidem affinity [292, 602]. Introduction of the point mutations γ_1 I79F, γ_1 L132M in γ_1 subunit and γ_3 L133M in γ_3 restores zolpidem affinity [292, 602]. A third region important for zolpidem affinity, was located recently downstream from these two residues in the Loop F of γ_2 subunit; residue γ_2 R194D was necessary for high affinity binding of the zolpidem, but not flunitrazepam [624].

12.27 MODULATION OF GABA_A RECEPTORS BY NEUROSTEROIDS, ANESTHETICS, ALCOHOLS, AND ANTICONVULSANTS

Neurosteroids [627–632], volatile [633], intravenous, and general anesthetics [634–641], alcohols [642–645], loreclezole, tracazolate, and related anticonvulsants [646–651] and allosteric antagonists can all modulate GABA_A receptor function [652–654] (see Fig. 12.22). Selye et al. [655] were the first to demonstrate that neuroactive steroids, their naturally occurring metabolites, and synthetic steroid derivatives can produce a rapid depression of CNS activity by nongenomic mechanisms [656, 657]. Neurosteroids are synthesized from cholesterol locally in brain cells, independent from their peripheral concentrations [632, 656, 658, 659]. 5α -Reductase transforms progesterone to 5α -dihydroprogesterone (5α -DPH), which can act on gene transcription via progesterone receptors of brain cells. 5α -DPH is reduced by 3α -hydroxysteroid oxidoreductase to allopregnanolone.

Neurosteroids acting at GABA_A receptors can be divided into two functional groups: uncharged, which act as positive allosteric modulators [199, 273, 660], and

TABLE 12.3 Binding Affinities (nM) of Benzodiazepine Site Ligands to GABA_A Receptors Containing Different γ Subunits as Measured by Radioligand Binding

Compound	$\alpha_1\beta_x\gamma_{1-3}$ ^a (Affinity, nM)		
	$\alpha_1\beta_x\gamma_1$	$\alpha_1\beta_x\gamma_2$	$\alpha_1\beta_x\gamma_3$
Chlordiazepoxide	—	890 ± 250 ^b	—
Diazepam	—	12 ± 1 ^b	180 ± 30 ^c
Flunitrazepam	37.74 ± 5.07 ^d	3.9 ± 0.5 ^b	150 ± 30 ^d
Triazolam ^d	25.65 ± 7.52	0.45 ± 0.13	6.04 ± 0.29
Bretazenil	—	0.22 ± 0.02 ^b	3.3 ± 0.5 ^e
Abecarnil	—	1.3 ± 0.2 ^b	—
Ro15-1788	> 5000 ^d	1.1 ± 0.04 ^b	2.80 ± 1.01 ^d
Ro15-4513	—	4.8 ± 0.1 ^b	2.8 ± 0.9 ^f
Zopiclone ^g	—	42.4 ± 2.5	135 ± 40
Zolpidem	> 5000 ^d	23 ± 5 ^b	2670 ± 350 ^c
Zaleplon	—	710 ± 160 ^h	—
FG8094	—	48.5 ⁱ	—
FG8205	—	0.9 ± 0.2 ^b	—
CGS9895	—	0.32 ± 0.05 ^b	—
CGS8216	—	0.17 ± 0.01 ^b	—
CL218872	> 5000 ^d	66 ± 0.3 ^b	6.3 ± 0.8 ^c
Ocinaplon	—	5367 ± 108 ^j	2220 ± 11 ^j
β -CCM	> 5000 ^d	2.4 ± 0.3 ^b	11 ^k
β -CCT	—	0.72 ± 1 ^l	—
DMCM	—	10 ± 0.7 ^b	9.8 ± 1.1 ^m
SL651,498	—	17 ± 1.5 ^l	—
L-655,708	—	48.5 ± 6 ⁿ	—
L-838,417	—	0.79 ± 0.18 ^o	—

^aReceptors were immunoprecipitated or expressed in clonal cell lines.^bFrom [583].^cFrom [292].^dFrom [602].^eFrom [520].^fFrom [620].^gFrom [621].^hFrom [549].ⁱFrom [610].^jFrom [759].^kFrom [150].^lFrom [611].^mFrom [615].ⁿFrom [554].^oFrom [551].

TABLE 12.4 Potency (nM) and Efficacy of Positive (% Enhancement) and Negative (% Inhibition) Modulators of GABA_A Receptors Containing Different Types of γ Subunit were Measured Using Electrophysiology

Compound	$\alpha_1\beta_X\gamma_{1-3}$ (Potency, nM/efficacy, %)		
	$\alpha_1\beta_X\gamma_1$	$\alpha_1\beta_X\gamma_2$	$\alpha_1\beta_X\gamma_3$
Diazepam	—	—	1950, +90 ± 8% ^d
Flunitrazepam	—	+156 ± 10 ^e 29 ± 11.2, +100 ± 8.9% ^e	110.7 ± 14.3, +88 ± 2.3% ^f
Triazolam	—	46 ± 10.2, +81 ± 3.4% ^e	—
Bretazenil	—	—	+170–230 ^g
Abecarnil ^f	+10 ± 1 ^b	+51, +94% ^h	~ +60% ^h
Rol15-1788	—	+74 ± 20%	+96 ± 28%
Rol15-4513	+4 ± 3% ^b	—	—
Zopiclone ^j	—	0% ⁱ –22 ± 7% ^e	—
Zolpidem	—	42.4 ± 2.5%, +284 ± 3.1% 57 ± 5.7% ^e	135 ± 40%, +419 ± 25%, 36500 ± 1928, +165% ^f
Zaleplon	+13 ± 3% ^b	+133 ± 31% ^e 499 ^k , +329% ⁱ	—
FG8094	—	—	—
FG8205	—	–12 ± 2% ^c 10 ± 1.1, +54 ± 1.4% ^e	—
CL218872	—	68 ± 28.9, +51 ± 5.3% ^e	—
Ocinaplon ^d	+6 ± 5% ^b	3070, +88 ± 8.6%	+66 ± 3 ^f 10030, +51 ± 3.5%

Continued

TABLE 12.4 (Continued)

Compound	$\alpha_1\beta_x\gamma_{1-3}$ (Potency, nM/efficacy, %)		
	$\alpha_1\beta_x\gamma_1$	$\alpha_1\beta_x\gamma_2$	$\alpha_1\beta_x\gamma_3$
β -CCM	—	—	—
β -CCT	—	— 39% ^m	—
	—	—	—
DMCM	—	— 5% ⁿ	—
	—	1 \pm 0.4 ^e	9.8 \pm 1.1,
	+ 8 \pm 4% ^b	— 71 \pm 2.9% ^o	— 63 \pm 1.3 ^f
SL651,498	—	—	—
L-655,708	—	\sim + 40%	—
	—	—	—
	— 7 \pm 2% ^b	—	—

^aReceptors were expressed in *Xenopus* oocytes or clonal cell lines.

^bFrom [622].

^cFrom [610].

^dFrom [385].

^eFrom [613].

^fFrom [615].

^gFrom [618].

^hFrom [528].

ⁱFrom [374].

^jFrom [621].

^kFrom [619].

^lFrom [619].

^mFrom [616].

ⁿFrom [374].

^oFrom [566].

^pFrom [551].

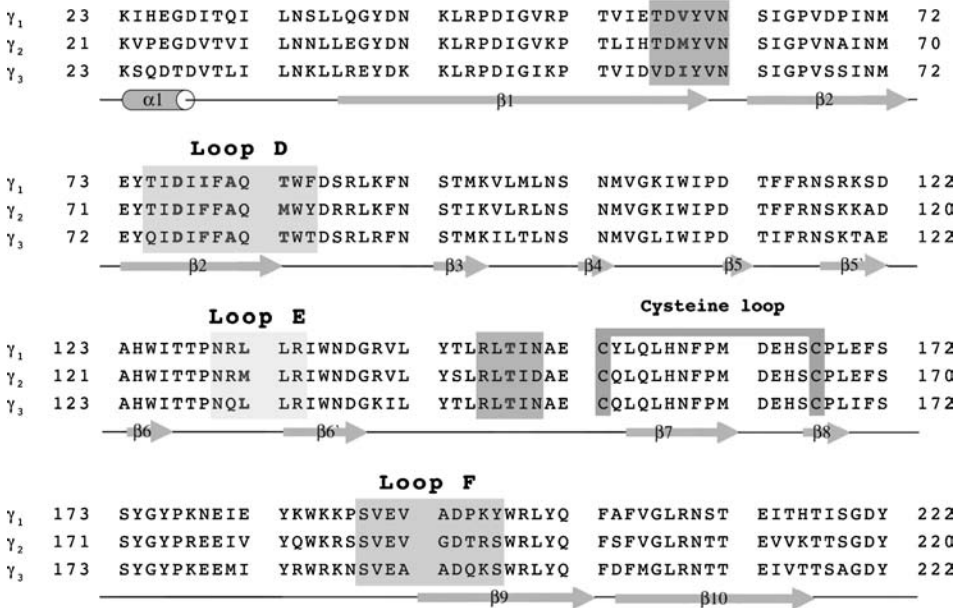


Figure 12.22 Alignments of the ligand binding domains of the GABA_A receptor γ subunits. Residues forming the cysteine loop are highlighted in grey. The colored boxes are regions known in the six-loop agonist binding site model: loops D (blue), E (cyan), F (dark green). Pink boxes contain residues that are not assigned to any “loops” but are important for benzodiazepine binding or allosteric modulation. Residues thought to be implicated in the formation of the benzodiazepine binding site are in bold blue. Precursor peptide sequences of human subunits were obtained from NCBI (access codes: γ_1 : NP_775807.2; γ_2 : P18507; γ_3 : Q99928). Ligand binding domains were aligned to the mature peptide sequence of the γ_1 subunit using MaxHom [744] at Predict Protein (<http://www.predictprotein.org/>). (See color insert.)

charged, negative allosteric modulators of receptor function [629–631, 661]. Enhancement of submaximal GABA_A receptor currents occurs through an increase in both channel open frequency and open duration [271, 273, 274, 628]. Charged neurosteroids inhibit GABA-gated channel openings by enhancing receptor desensitization and stabilizing desensitized states [662, 663].

Pregnanolone and allopregnanolone have been associated with anxiolytic effects in mice on the elevated plus-maze test [664–666]. Synthetic analogs of neuroactive steroids have been investigated for potential use as anxiolytics, anesthetics, and anticonvulsants [545, 657, 667, 668].

Ganaxolone (3-hydroxy-3-methyl-5-pregnan-20-one) is an orally active synthetic analog of allopregnanolone and a positive allosteric modulator of GABA_A receptor [545, 667, 669]. Ganaxolone is a more effective blocker of the anxiogenic effects induced by pentylenetetrazol in mice than diazepam [670]. Wieland et al. [671] have reported that 3 β -substituted analogs of the endogenous neuroactive steroid 3 α -hydroxy-5 α -pregnan-20-one (e.g., 3 β -ethenyl-3 α -hydroxy-5 α -pregnan-20-one, or Co 3-0593) exhibit anticonvulsant activity against pentylenetetrazol-induced seizures in mice and rats (median effective dose ED₅₀ values of 5.6 and 11.5 mg/kg i.p.,

respectively). In addition, Co 3-0593 produces anxiolytic effects in the Geller–Seifter test, comparable to benzodiazepines. Furthermore, the anxiolytic activity of Co 3-0593 remains even after chronic administration, suggesting an absence of tolerance. This compound does not affect the acquisition of a learned response at both anticonvulsant and anxiolytic doses. However, at higher doses it induces rotarod performance deficits that are further enhanced by ethanol [671]. These findings suggest that synthetic neurosteroids that can easily cross the blood–brain barrier may represent a novel class of potentially useful therapeutic agents [666, 672, 673].

GABA_A receptors are particularly sensitive to many anesthetic agents, and there is a strong correlation between the concentrations of these compounds that affect receptor function and their anesthetic potencies [674–676]. A wide range of compounds modulate GABA_A receptors, including volatile anesthetics such as isoflurane [677–680], halothane [674], and enflurane [676, 681], as well as nonvolatile agents such as pentobarbital, etomidate, etifoxine, and propofol [679, 680, 682, 683]. These compounds can, at high concentrations, directly gate the ion channel of the GABA_A receptor in the absence of GABA [631, 684–686]. At low concentrations they modulate GABA-induced openings [636], and, depending on the type of compound, this potentiation of GABA-gated currents appears to alter receptor activation/deactivation and/or desensitization [274, 687–689].

Small alcohols such as ethanol and trichloroethanol might bind to a domain on or associated with GABA_A receptors and produce anxiolytic and hypnotic effects [690]. Ethanol appears to interact more readily at physiologically relevant concentrations with receptors containing a δ subunit [690]. Interestingly, the hypnotic effects and potencies of the *n*-alcohols increase with carbon chain length. Thus, *n*-hexanol and *n*-octanol produce greater effects at lower concentrations than does ethanol [691].

Behavioral studies have shown that loreclezole, tracazolate, etazolate, and etifoxine, compounds structurally unrelated to benzodiazepines and neurosteroids, possess anxiolytic and anticonvulsant activities [646–651, 692–694]. Compared with the standard benzodiazepine chlordiazepoxide, these compounds are 2–20 times less potent as anxiolytics but, surprisingly, display a much larger window of separation between the anxiolytic effect and potential side effects (sedation, motor incoordination, and its interaction with ethanol and pentobarbital) in mammals [650, 693]. Binding and electrophysiological experiments have demonstrated that these compounds bind to GABA_A receptors via an allosteric site that differs from the benzodiazepine and neurosteroid modulatory sites [250, 694–697].

12.28 ALLOSTERIC SITES WITHIN TRANSMEMBRANE DOMAIN OF RECEPTOR SUBUNITS

A large body of evidence has been collected suggesting that neurosteroids, anesthetics, barbiturates, alcohols, and a number of other drugs share overlapping structural determinants for their actions on the GABA_A receptor [701].

A set of residues located in the TM 1–4 of GABA_A receptor α and β subunits confers potency to various clinically used compounds (Figs. 12.23 and 12.24). Residues implicated in the formation of these binding sites are located within homologous domains of α and β subunits. It is worth noting that the same

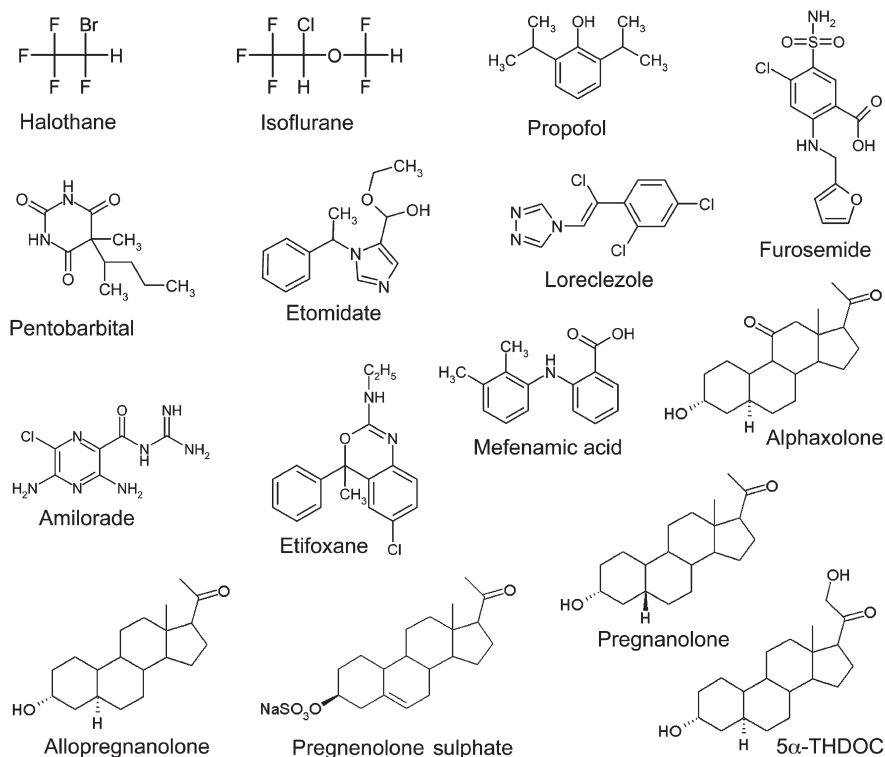


Figure 12.23 Compounds acting via binding sites located within transmembrane domains of GABA_A receptor subunits. Volatile anesthetics (halothane and isoflurane), general anesthetics (propofol, pentobarbital, and etomidate), anticonvulsants with anxiolytic properties (lorelezole, mefenamic acid, etifoxine), allosteric antagonists (furosemide and amiloride), and neurosteroids [pregnanolone, allopregnanolone, pregnenolone sulfate (PS) and 5 α -THDOC].

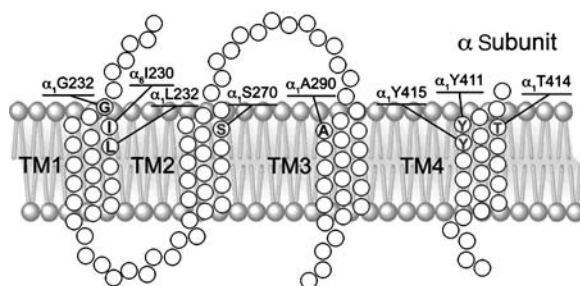


Figure 12.24 Schematic view of the membrane part of GABA_A receptor α subunit with residues implicated in formation of different allosteric binding sites.

transmembrane regions have been described as integral parts of the channel gating domain of the GABA_A receptor [128, 434] and other ligand-gated ion channels [223, 224, 277, 442].

Mutation of residue α_1 G223F located on the TM1 segment of α subunits reduces gating induced by pentobarbital and propofol [745]. Mutation of another residue, α_2 L232F, which is also located on TM1 reduces potentiation by halothane [698]. It was also found that a single amino acid in the α_6 -subunit TM1 segment, α_6 I230, confers sensitivity to furosemide inhibition [653, 699].

In the TM2 and TM3 segments α_1 S270 and α_1 A291 residues are part of the binding site for ethanol [643, 645, 700], halothane, isoflurane, and propofol [702–707] (Fig. 12.23). Mutation of these residues greatly reduces potentiation of GABA-gated currents by these compounds. Interestingly, the α_1 -subunit TM4 segment harbors an additional set of complementing residues of this allosteric site. Introduction of a tryptophan mutation in residues α_1 Y411, α_1 T414, and α_1 Y415 reduces the ability of isoflurane, halothane, and chloroform to potentiate GABA-gated currents [705].

Residues β_2 G219F, β_2 N265, and β_2 M286 located on TM domains 1–4, which are homologous to α_1 G232, α_1 S270, and α_1 A291, confer sensitivity to inhaled, general anesthetics and anticonvulsants [637, 638, 640, 641, 647, 651, 653, 700, 708–710] (Fig. 12.24).

Mice with β_2 N265S knockin mutation lack the sedative effects produced by etomidate [369] whereas the β_3 N265S mutation renders mice insensitive to the anesthetic effects of propofol and etomidate, with a small reduction in potency of volatile anesthetics [370].

Selectivity of loreclezole for β_2/β_3 - over β_1 -subunit-containing receptors is determined by the TM3 residue β_2 N289/ β_3 N290. Mutating a single serine to asparagine in the β_1 subunit (β_1 S289N) is sufficient to confer loreclezole sensitivity to β_1 -containing receptors [711].

Residues conferring sensitivity to GABA_A receptors for Zn^{2+} were identified in the TM2 domain. Mutations of residues β_2 H267 and β_2 G270 located close to the entrance of the channel pore reduce zinc inhibition about 650-fold [712–714].

Recent findings suggest that the action of some neurosteroids on GABA_A receptor is mediated through sites formed by the TM part of receptor subunits. Thus, it was discovered that modulation of ρ -containing receptors by allotetrahydrodeoxycorticosterone (5 α -THDOC), 5 β -pregnane-3 α -ol-20-one (pregnanolone), and 5 β -dihydroprogesterone (5 β -DHP) is dependent on residue ρ_1 I307, which is homologous to β_2 N265/ α_1 S270 [715]. Experiments using α_1/ρ_1 chimeric receptors indicate that there is a region from TM4 to the C-terminus of the GABA_A receptor α_1 subunit that is critical for the effects of neurosteroids [716].

12.29 CHANNEL BLOCKERS AND THEIR BINDING SITE

Channel blockers antagonize GABA-elicited currents in a noncompetitive fashion [717–721] and act as convulsants in vivo (Fig. 12.25). Picrotoxinin, U-93631, TBPS, and some insecticides are thought to bind at a single binding site located within the channel pore [468, 469, 722–724].

The binding site of channel blockers is formed by residues located on TM2 segments of both α and β subunits (Fig. 12.26). Residues conferring sensitivity to picrotoxinin and TBPS are α_1 V257 and α_1 T261 [722, 723] and β_2 A252 and β_2 L253 (Fig. 12.27) [468, 469]. The β_2 T246 located on the linker between TM1 and TM2 segments affects the potency of the convulsant compound pentylenetetrazole [724].

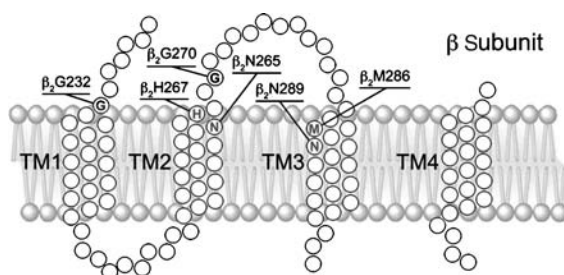
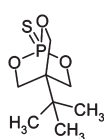
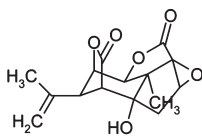


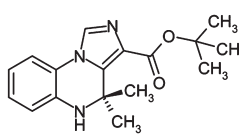
Figure 12.25 Schematic view of the membrane part of GABA_A receptor β subunit with residues implicated in formation of different allosteric binding sites.



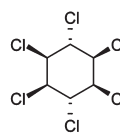
TBPS



Picrotoxinin



U-93631



Lindane

Figure 12.26 Chemical structure of some channel blocker of GABA_A receptors.

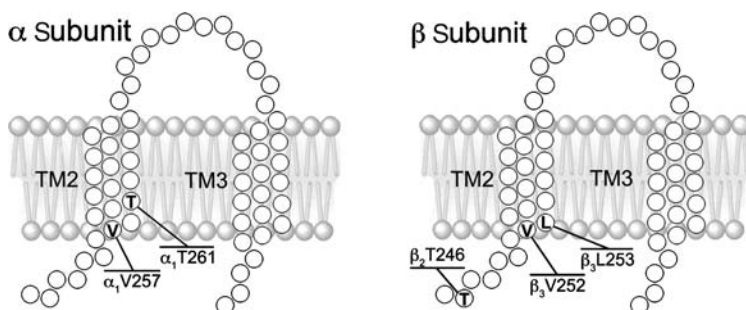


Figure 12.27 Schematic view of the membrane domains 2 and 3 of α and β subunits of the GABA_A receptor with residues forming binding site for the channel blockers.

12.30 MODULATION OF GABA_A RECEPTORS VIA UNIDENTIFIED ALLOSTERIC SITES

The structural determinants of the recognition sites for a number of compounds that allosterically modulate or act through GABA_A receptors directly have not been identified thus far.

The γ -butyrolactones are a class of compounds that interact with the GABA_A receptor at a site different from those of benzodiazepines or barbiturates [725, 726]. Competition binding experiments using [³⁵S]TBPS as a radioligand suggest that γ -butyrolactones bind at the picrotoxinin site [727–729]. However, the potentiation of GABA responses by these compounds is retained at receptors containing a mutation that disrupts the picrotoxinin recognition site [730–732].

A number of fatty and unsaturated acids modulate GABA_A receptor function. Arachidonic, eicosatetraenoic, and oleic acids inhibit currents elicited by GABA and muscimol in brain preparations and recombinant GABA_A receptors in a dose-dependent manner [733–735]. Thyroid hormones such as L-triiodothyronine (T₃) and L-thyroxine are also reported to interact with GABA_A receptors [736], and it has been suggested that the α_1 subunit imparts T₃ sensitivity [736]. The antihelminthic compound avermectin [737, 738], the anxiolytic anticonvulsant compounds chlormethiazole and trichloroethanol [739–741], polyamines such as spermine and spermidine [742], and antidepressants such as amoxapine and mianserin [743] have been reported to interact with GABA_A receptors, but the exact site of action of these drugs and their subunit requirements are not known.

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13

METABOTROPIC GABA RECEPTORS

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13.1	Introduction	570
13.2	Receptor Structure	571
13.2.1	Expression Cloning of GABA _B Receptors	571
13.2.2	Molecular Subtypes	572
13.2.3	Surface Trafficking and Heteromerization	575
13.2.4	Ligand Binding Site	578
13.2.5	Molecular Determinants of G-Protein Coupling	579
13.2.6	Allosteric Interactions Between Receptor Subunits	580
13.3	Effector Systems	580
13.3.1	G-Protein-Dependent and G-Protein-Independent GABA _B Effects	580
13.3.2	Ca ²⁺ Channels	581
13.3.3	K ⁺ Channels	582
13.3.4	Adenylate Cyclase	582
13.3.5	MAPK	583
13.4	GABA _B Receptor Modulation	584
13.4.1	Extracellular Calcium	584
13.4.2	Phosphorylation and Receptor Desensitization	584
13.4.3	Interacting Proteins	585
13.5	GABA _B -Deficient Mice	587
13.6	Pharmacology	588
13.6.1	Endogenous GABA _B ligands: GABA and GHB	588
13.6.2	Agonists and Competitive Antagonists	589
13.6.2.1	Agonists	589
13.6.2.2	Antagonists	590
13.6.3	Novel GABA _B Compounds	590
13.6.3.1	Allosteric Modulators	590
13.6.3.2	Subtype-Selective Ligands	592
13.7	Disease	592
13.7.1	GABA _B Receptors as Therapeutic Targets	592
13.7.2	Addiction	593

13.7.3	Anxiety and Depression	594
13.7.4	Epilepsy	595
13.7.5	Nociception	595
13.7.6	Tumor Cell Growth and Migration	596
13.7.7	Genetic Linkage Studies	596
	References	597

13.1 INTRODUCTION

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS) and as such plays a key role in modulating neuronal activity. GABA mediates its action via distinct receptor systems, the ionotropic GABA_A and metabotropic GABA_B receptors. Unlike GABA_A receptors that form ion channels, GABA_B receptors address second-messenger systems through the binding and activation of guanine-nucleotide binding proteins (G proteins). Hence, the effects of activating GABA_B receptors are slower and longer lasting than those of activating GABA_A receptors. GABA_B receptors were first demonstrated on presynaptic terminals where they inhibit neurotransmitter and neuropeptide release by suppressing a Ca^{2+} conductance or by exerting a direct effect on the release machinery. Subsequent studies also showed the presence of GABA_B receptors at postsynaptic sites where they inhibit neuronal excitability by increasing a K^{+} conductance. Therefore, GABA_B receptors can have either excitatory or inhibitory effects on the overall excitability in a neuronal network, depending on their cellular/subcellular localization and the temporal/spatial pattern of activation relative to other synaptic inputs.

Given this multitude of GABA_B functions it is not surprising that dysfunction of GABA_B-mediated synaptic transmission in the CNS is believed to underlie various nervous system disorders. For example, GABA_B receptors were linked to epilepsy, anxiety, sleep disorders, depression, addiction, and pain. GABA_B research, because of its medical relevance, has always attracted a great deal of attention in academia and industry. The prototypic GABA_B receptor agonist baclofen (β -chlorophenyl-GABA, Lioresal) was introduced to the market in 1972 and is still used to treat spasticity and skeletal muscle rigidity in patients with spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, and cerebral palsy. In addition, the partial GABA_B agonist γ -hydroxybutyrate (GHB) (Xyrem) has recently been approved in different countries for such varied uses as general anaesthesia, the treatment of alcohol withdrawal and addiction, and, most recently, cataplexy associated with narcolepsy. Baclofen and other GABA_B agonists showed promising therapeutic effects in a whole range of other indications, but their side effects, including sedation, tolerance, and muscle relaxation, prevented further development. Many researchers in the field assumed that a dissociation of the therapeutic effects from the side effects would be achievable with more selective GABA_B drugs. This assumption was based on a large body of literature suggesting the existence of pharmacologically distinct GABA_B receptor subtypes in the brain. However, molecular cloning efforts resulted

in the identification of only three abundant GABA_B receptor complementary deoxyribonucleic acids (cDNAs), derived from two genes, *GABA_{B1}* and *GABA_{B2}*. Molecular heterogeneity in the GABA_B system is provided by the GABA_{B1} isoforms GABA_{B1a} and GABA_{B1b} that are generated from the same gene by alternative promoter usage. GABA_{B1a} and GABA_{B1b} differ in their extracellular domain and each assembles with the GABA_{B2} subunit to form pharmacologically similar heterodimeric GABA_{B(1a,2)} and GABA_{B(1b,2)} receptors. Much is now known about the roles of the individual subunits in the activation, assembly, and signaling of the heterodimer, which will be discussed in detail below. The existence of only two abundant molecular subtypes of GABA_B receptors was a surprise to many in the field who expected a variety of pharmacologically distinct GABA_B receptors, as predicted from the work on native receptors. Efforts therefore turned toward identifying GABA_B receptor-associated proteins, receptor modifications, and endogenous factors that possibly were responsible for generating pharmacological differences. We provide an overview on the current knowledge on protein interactions with GABA_B receptors. With the puzzling lack of pharmacologically distinct receptor subtypes, it became important to understand to which known GABA_B functions the cloned subunits contribute in vivo. To this end a number of laboratories disabled GABA_B genes in mice by gene targeting. Analysis of mice deficient for *GABA_{B1}* or *GABA_{B2}* confirmed that both GABA_B subunits are critical for the classical GABA_B responses in vivo. However, it also revealed that functional GABA_B receptors could exist in neurons that naturally lack GABA_{B2} subunits, which may add to the molecular and functional diversity of GABA_B receptors. A long-standing question in the field concerns the relationship between GABA_B receptors and the receptors for GHB, a metabolite of GABA and emerging drug of abuse. It is a matter of much debate whether specific GHB receptors exist and whether they are related to GABA_B receptors. This question was addressed using molecular tools and GABA_B knockout mice. Furthermore, we review recent studies that tried to establish a link between polymorphisms in GABA_B genes and congenital human diseases. Taking recent developments in the field into account, we also touch on the most promising indications for GABA_B drugs. Last but not least, the cloned receptors were used to establish high-throughput compound screens based on functional assays, which yielded the first allosteric compounds acting at GABA_B receptors. The features of these compounds will be discussed.

13.2 RECEPTOR STRUCTURE

13.2.1 Expression Cloning of GABA_B Receptors

It took close to 20 years after their discovery by Bowery and colleagues [1, 2] until GABA_B receptors were cloned. As with many neurotransmitter receptors, biochemical isolation was hindered by the lack of ligands that bind the receptor under solubilizing conditions and cell lines that express significant amount of receptor protein. Ultimately expression cloning using a high-affinity GABA_B radioligand resulted in the identification of two closely related cDNAs [3]. The two cDNAs derive from a single gene by alternative promoter usage and encode proteins of 960 and

844 amino acids, designated GABA_{B1a} and GABA_{B1b}. Recombinantly expressed GABA_{B1a} and GABA_{B1b} proteins have molecular masses of 130 kDa and 100 kDa, respectively, which are similar to the ones reported for native receptors. Sequence comparison revealed that GABA_B receptors belong to the family 3 (also named family C) of G-protein-coupled receptors (GPCRs), which also includes the metabotropic glutamate receptors (mGluRs), Ca²⁺-sensing receptors (CaS), vomeronasal receptors, taste receptors, and a number of orphan GPCRs.

While GABA_{B1} subunits showed many of the expected features of native GABA_B receptors in terms of structure and distribution, they surprisingly did not efficiently couple to their effector systems [4]. Moss and colleagues were the first to show that GABA_{B1} proteins are not transported to the cell surface but remain associated with the endoplasmic reticulum (ER) when expressed in heterologous cells [5]. This was taken to explain the 100- to 150-fold lower affinity for agonists that is observed with recombinant GABA_{B1} subunits when compared to native GABA_B receptors [3]. Presumably the failure to traffic to the cell surface prevents the interaction of GABA_{B1} subunits with G proteins in the plasma membrane, which is necessary to stabilize the high-affinity conformation of the binding site [6]. Ultimately, the identification of a second GABA_B subunit termed GABA_{B2} provided the necessary explanation [7–12]. In heterologous cells GABA_{B2} must be coexpressed with GABA_{B1a} or GABA_{B1b} to form a fully functional receptor. The GABA_{B2} subunit not only serves to escort GABA_{B1} to the cell surface but also appears to be the receptor component that mainly links to the G protein. This finding represented the first compelling evidence for heterodimerization among GPCRs. Until then it was thought that functional GPCRs mainly exist in the plasma membrane as monomers or homodimers. Recombinant heteromeric GABA_{B(1,2)} receptors couple to all prominent effector systems of native GABA_B receptors, that is, adenylate cyclase, Kir3-type K⁺ channels, and P/Q and N-type Ca²⁺ channels [13–15]. Moreover, when the GABA_{B2} subunit is coexpressed with GABA_{B1}, agonist potency more closely approximates that of native receptors. A 10-fold lower affinity compared to brain receptors is still observed for recombinant GABA_{B(1,2)} receptors, which may be explained by limiting amounts of the G protein in the heterologous cells [8].

13.2.2 Molecular Subtypes

When it became apparent that probably all GABA_B receptors in the vertebrate brain are the sole products of the *GABA_{B1}* and *GABA_{B2}* genes, much attention focused on subunit isoforms generated by alternative splicing. Many in the field wondered whether such isoforms encoded pharmacological differences and accounted for the heterogeneity observed with native GABA_B receptors. Rapidly numerous GABA_B splice variants were identified (recently reviewed in [16]). A close inspection of the gene structures indicates that not all proposed splice variants are real and that some do not occur across different species. While in most laboratories mixing and matching of splice variants did not produce GABA_B receptors with distinct functional and pharmacological properties, others reported differences that are, however, highly controversial. GABA_B isoforms may afford the means for a differential subcellular compartmentalization and/or coupling to distinct intracellular signaling pathways. To some extent a coupling to different effector systems could

mimic differential agonist pharmacology and explain some of the differences that were observed with native GABA_B receptors.

The most abundant GABA_B receptor isoforms are GABA_{B1a} and GABA_{B1b}, which exhibit dissimilarity in the extracellular domain (ECD) [3]. The GABA_{B1a} and GABA_{B1b} isoforms are the only variants that are highly conserved among different species. The first 147 amino acids of the mature GABA_{B1a} isoform are replaced in GABA_{B1b} with a sequence of 18 amino acids. Contrary to the general assumption, GABA_{B1a} and GABA_{B1b} are not generated by N-terminal alternative splicing. The distinct ECD in GABA_{B1b} results from the presence of an alternative transcription initiation site within the GABA_{B1a} intron upstream of exon 6, thereby extending exon 6 at its 5' end (Fig. 13.1) [17, 18]. Presumably, GABA_{B1a} and GABA_{B1b} use different promoters, with the GABA_{B1b} promoter being buried within GABA_{B1a} intron sequences (Fig. 13.2). Indeed, it was recently shown that multiple DNA regulatory elements, identified in the 5'-flanking regions, selectively regulate expression of GABA_{B1a} and GABA_{B1b} transcripts [19]. Alternative N-termini are rather exceptional for GPCRs and are not observed in any of the closely related family 3 GPCRs. Structurally, GABA_{B1a} differs from GABA_{B1b} by a pair of extracellular "sushi repeats," a common protein interaction module originally identified in proteins of the complement system. Sushi repeats, or SCRs (short consensus repeats) as they are also referred to, have yet to exhibit a function in the context of the GABA_B receptor. It is tempting to speculate that GABA_{B1a} is compartmentalized to specific subcellular regions by means of interaction of its sushi repeats with proteins in the neuron itself, in the extracellular matrix, or on the surface of neighboring cells. It was proposed that the GABA_{B1a} sushi repeats interact with the extracellular matrix protein fibulin; however, a physiological relevance for this interaction has yet to be demonstrated [20]. A recent report showing that the ECD 1 of a type B1 GPCR folds into a sushi repeat suggests that these protein-protein interaction modules are more common among GPCRs than previously thought [21].

Numerous studies indicate that GABA_{B1a} and GABA_{B1b} show differences in their spatial and temporal expression patterns [3, 8, 13, 22–29]. A pre- versus postsynaptic localization was suggested for both isoforms but due to the lack of selective pharmacological tools was never directly demonstrated [13, 23, 28]. A striking example of a differential expression of GABA_{B1a} and GABA_{B1b} is found in the cerebellum (Fig. 13.1) [13, 24]. GABA_{B1a} transcripts are mostly confined to the granule cell layer that comprises the cell bodies of the parallel fibers, which are excitatory to the Purkinje cell dendrites in the molecular layer. By comparison GABA_{B1b} transcripts are mostly expressed in Purkinje cells, the dendrites of which possess GABA_B receptors that are postsynaptic to GABAergic Basket and Stellate cells or glutamatergic parallel fibers. In dorsal root ganglia the density of GABA_{B1a} transcripts is high as opposed to GABA_{B1b} transcripts [28]. GABA_{B1b} protein is generally expressed at higher levels in the adult brain when compared to fetal brain, whereas the opposite is seen during development (Fig. 13.1) [18, 22, 25, 30]. These spatial and temporal differences in the expression of the GABA_{B1a} and GABA_{B1b} subunits highlight the separate transcriptional regulation and suggest distinct functional roles. A number of laboratories compared the pharmacological properties of GABA_{B1a} and GABA_{B1b} without detecting significant differences [8, 22, 31, 32]. However, there are isolated reports that claim that GABA_{B1a} and GABA_{B1b} can be separated by pharmacological or functional means [33–35]. Especially, the proposal

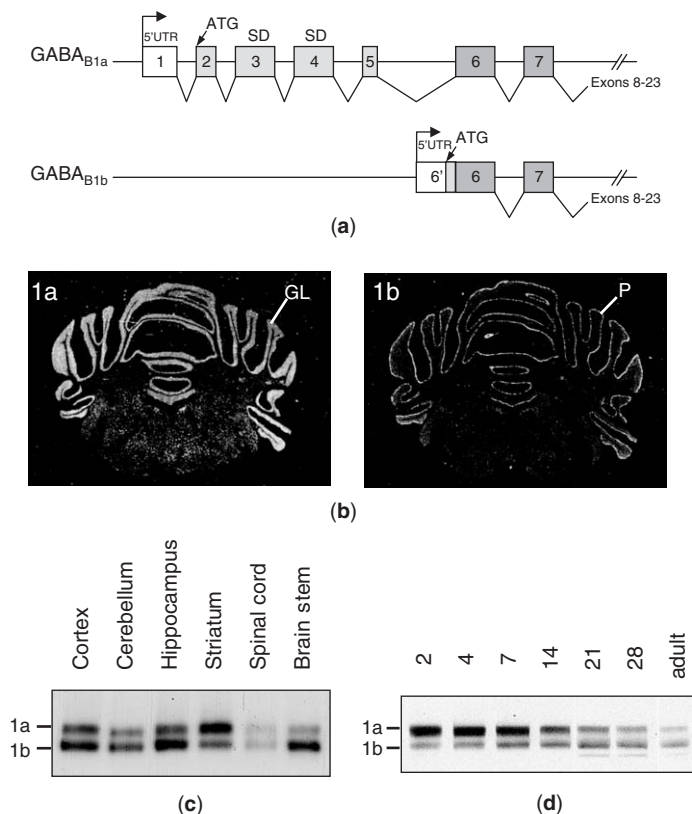


Figure 13.1 Differential expression of GABA_{B1a} and GABA_{B1b} transcripts. (a) Differential promoter usage results in generation of GABA_{B1a} and GABA_{B1b} transcripts. The GABA_{B1b} isoform uses an alternative transcription initiation site within the GABA_{B1a} intron upstream of exon 6. Thus the first 147 amino acids of the mature GABA_{B1a} isoform are replaced in GABA_{B1b} with a sequence of 18 amino acids. The amino terminal extracellular domains of GABA_{B1a} and GABA_{B1b} primarily differ by the presence of a pair of sushi domains (SDs) encoded by GABA_{B1a} exons 3 and 4. Exons are indicated as boxes (not drawn to scale). The transcription start sites (arrows) and translation initiation codons (ATG) are labeled. (b) Spatial distribution of GABA_{B1} transcripts in the cerebellum using isoform-specific riboprobes. GABA_{B1a} transcripts are predominantly observed in the granule cell (GL) layer, whereas GABA_{B1b} mRNA is present in the Purkinje cells (P). (c) Photoaffinity crosslinking of GABA_B receptor proteins. GABA_{B1a} and GABA_{B1b} are differentially expressed throughout the CNS. (d) Developmental expression of GABA_B receptors. GABA_{B1b} protein is generally expressed at higher levels in the adult brain compared with fetal brain, whereas GABA_{B1a} protein is more abundant during development. Numbers refer to postnatal days. (See color insert.)

that the anticonvulsant gabapentin is an agonist at GABA_{B(1a,2)}, but not at GABA_{B(1b,2)}, attracted a great deal of attention [33, 34, 36]. Not only was gabapentin suggested to be subunit specific, but it was also shown to selectively activate postsynaptic GABA_B receptors. This remains highly controversial, as a number of other laboratories were unable to reproduce these findings using similar and additional experimental approaches [37–39]. In these latter studies, no clear effect

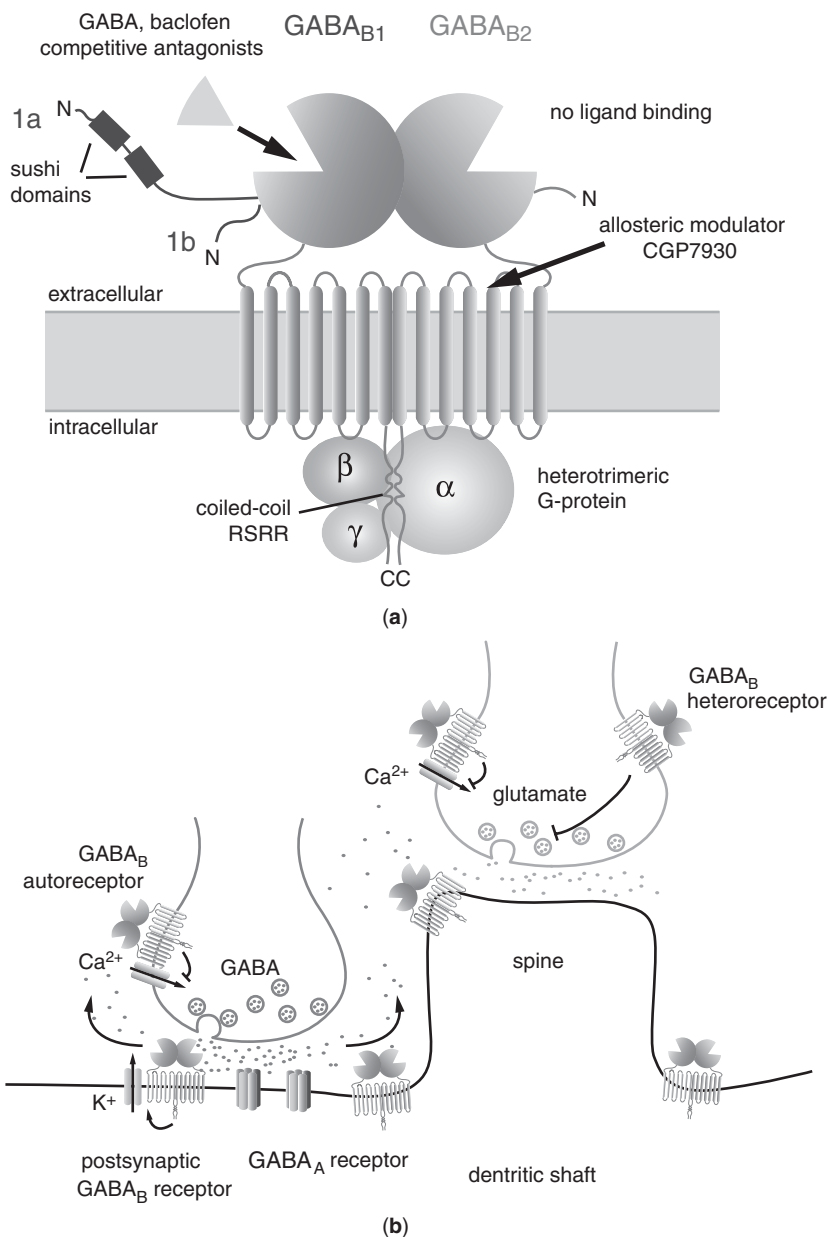
of gabapentin on GABA_B receptors was seen in recombinant systems, in brain slice preparations or in vivo, even when using high concentrations of the drug.

Besides GABA_{B1a} and GABA_{B1b}, the existence of several additional GABA_{B1} isoforms was reported. However, genomic analysis only confirmed splice junctions consistent with the formation of GABA_{B1c} and GABA_{B1e} by exon skipping from a parent GABA_{B1a} transcript [18]. Whereas skipping of exon 4 in GABA_{B1c} would result in a protein lacking the second extracellular sushi domain, skipping of exon 11 in GABA_{B1e} would produce a truncated secreted GABA_{B1a} fragment through the introduction of a frame-shift stop codon in the ECD. The existence of protein expressed in vivo has not yet been demonstrated for any of these additional GABA_{B1} splice variants. With regard to GABA_{B2}, the available data strongly support that only the initially described transcript is expressed in vivo. Hence, it is now generally accepted that the molecular distinction in the GABA_B system is predominantly based on the subunit isoforms GABA_{B1a} and GABA_{B1b}, which each assemble with the GABA_{B2} subunit to form heterodimeric GABA_{B(1a,2)} and GABA_{B(1b,2)} receptors.

13.2.3 Surface Trafficking and Heteromerization

As the function of GABA_B receptors critically depends on heterodimerization, a quality control mechanism has evolved that prevents trafficking of unassembled GABA_{B1} subunits to the cell surface. It is now well established that this involves an ER retention/retrieval signal, the four amino acids RSRR, within the cytoplasmic tail of GABA_{B1} [5, 40, 41]. ER retention/retrieval signals of this type were also observed in a number of other multisubunit proteins, for example, the K_{ATP} channels [42] and *N*-methyl-D-aspartate (NMDA) receptors [43], where they control stoichiometry and surface expression of the channel complex. Similarly, it is thought that association of GABA_{B1} with GABA_{B2} masks its ER retention/retrieval signal, allowing for the functional heterodimer to exit the ER. However, the exact mechanism for ER anchoring and release is still unclear (see below). Within the RSRR motif of GABA_{B1} the serine residue and the third arginine are not absolutely critical for function, as they can be substituted by other amino acids [5, 40, 41]. It was proposed that the functionality of the RSRR motif of GABA_{B1} depends on surrounding sequences [44]. In particular, amino acid residues that are part of the coiled-coil domain and neighbor the RSRR motif N terminally were shown to be important for recognition of the RSRR motif. From these experiments it was concluded that the minimal ER retention sequence in GABA_{B1} is comprised of the amino acids QLQXRQQLRSRR, where X can be either S or D [44]. However, it was recently shown that there is not an absolute requirement for the RSRR motif to be in its normal sequence context and that primarily appropriate spacing to the plasma membrane is important for its function [45]. The ER retention/retrieval signal in GABA_{B1} is inactivated through interaction with the C-terminus of GABA_{B2}, allowing correctly assembled GABA_{B(1,2)} heterodimers to leave the ER. Structural and mutational analysis identified α helices in the cytoplasmic tails of GABA_{B1} and GABA_{B2}, which were shown to form heterodimeric coiled-coil structure. However, cytoplasmic coiled-coil domain interaction is not an absolute requirement for shielding of the ER retention/retrieval signal in GABA_{B1} and surface trafficking of the heteromeric receptor complex [41]. Recent data suggest a model in which global conformational changes associated with heteromeric assembly remove the ER retention/retrieval signal from

the active zone, thereby restricting its access and triggering surface delivery of the complex [45]. COPI and 14-3-3 are prime candidates for regulating aspects of GABA_B receptor trafficking. COPI components can interact with arginine-based motifs and compete for binding with proteins of the 14-3-3 family [46]. It is thought that 14-3-3 binding overcomes ER retention by preventing recycling of correctly assembled proteins from the ER-Golgi intermediate compartment to the ER via COP1 vesicles [47, 48]. 14-3-3 proteins are known to associate with the C-terminus of



GABA_{B1} through a domain partially overlapping with the coiled-coil domain [49]. It is conceivable that COP1 components bind to RSRR when GABA_{B1} is unassembled, which recycles GABA_{B1} back to the ER. Following heteromeric assembly and removal of the RSRR motif from its active zone, COP1 could then be replaced by 14-3-3, which avoids recycling and allows for surface trafficking. Recently a di-leucine motif in the cytoplasmic tail of GABA_{B1} was identified that is thought to regulate receptor trafficking at the trans-Golgi network (TGN) level through interaction with a guanine-nucleotide exchange factor for the adenosine diphosphate (ADP) ribosylation factor (ARF) family of GTPases [50]. Therefore it was proposed that trafficking of GABA_{B1} subunits is regulated by two distinct motifs within the secretory pathway. Whereas the RSRR motif plays a role in ER retention or the recycling to the ER from the ER-Golgi intermediate compartment, the LL motif could regulate forward trafficking from the TGN to the plasma membrane. A recent study suggested that in addition to GABA_{B2} the γ 2S subunit of the GABA_A receptor can interact with GABA_{B1} and promote its transport to the cell surface [51]. Since GABA_{B2} not only escorts GABA_{B1} to the cell surface but also influences agonist affinity and G-protein coupling (see Section 13.2.6), the resulting GABA_B receptors are expected to differ in their functional and pharmacological properties from the heterodimeric GABA_{B(1,2)} receptors. Of note, it has been shown that GABA_{B2} knockout mice still exhibit electrophysiological GABA_B responses, showing that *in vivo* GABA_{B1} can to some extent overcome ER retention and function in the absence of GABA_{B2} [52]. However, at this point, it cannot be decided whether GABA_B receptors lacking a GABA_{B2} subunit are of physiological relevance or whether they represent an artifact of the knockout situation. Recently, an added level of complexity for the regulation

Figure 13.2 GABA_B receptor structure and subcellular localization. (a) Native GABA_B receptors are heterodimers of two subunits GABA_{B1} and GABA_{B2} (dark blue and pink, respectively). Heterodimerization and cell surface trafficking are facilitated through coiled-coil domain interaction within the cytoplasmic tails. A quality control mechanism involving an ER retention/retrieval signal, the four amino acids RSRR, within the cytoplasmic tail of GABA_{B1} prevents trafficking of unassembled GABA_{B1} to the cell surface. Molecular heterogeneity in the GABA_B system is provided by the GABA_{B1} isoforms GABA_{B1a} and GABA_{B1b}, which are generated by alternative promoter usage. Structurally, GABA_{B1a} differs from GABA_{B1b} by a pair of extracellular sushi repeats, a common protein interaction module. All known GABA_B receptor agonists and competitive antagonists bind to the GABA_{B1} subunits only, whereas G-protein activation is facilitated through the GABA_{B2} subunit. Mutagenesis studies and the lack of evolutionary conservation indicates that the extracellular domain of GABA_{B2} is not the binding site of a natural ligand. Recently, the binding site of the positive allosteric modulator CGP7930 has been localized to the transmembrane domain of GABA_{B2}. (b) In the CNS, GABA_B receptors are localized presynaptically, postsynaptically, and at extrasynaptic sites. Presynaptic GABA_B receptors are subdivided into those that control GABA release (autoreceptors) and those that control the release of other neurotransmitters (e.g., glutamate) and neuropeptides (heteroreceptors). This is achieved by suppressing a Ca²⁺ conductance or by exerting a direct effect on the release machinery (observed in glutamatergic synapses). Postsynaptic GABA_B receptors activate K⁺ channels and induce slow inhibitory postsynaptic potentials (IPSPs), the fast component of which is mediated through GABA_A receptors. Heteroreceptors and extrasynaptic receptors are likely to be activated through “spillover” GABA from adjacent GABAergic synapses. (Modified from [290].) (See color insert.)

of GABA_B receptor assembly and trafficking was suggested by the identification that Marlin-1, a novel RNA binding protein expressed in neurons [53]. Marlin specifically interacts with GABA_{B1} protein but controversially regulates cellular levels of GABA_{B2} in functional experiments.

Integrating these findings it is emerging that interaction of the cytoplasmic tails of GABA_{B1} and GABA_{B2} with each other and with proteins that modulate vesicular transport is crucial for regulating cell surface trafficking of GABA_B receptors. Surprisingly, however, the cytoplasmic tails are dispensable for heterodimerization as such. It was demonstrated that C terminally truncated GABA_{B1} and GABA_{B2} subunits can form fully functional receptors when coexpressed in heterologous cells [41]. This indicates that the transmembrane domains and/or ECDs encode surfaces that are sufficient for heteromerization. This is in agreement with data that suggest that other family 3 GPCRs (e.g., the mGlu1, mGlu5, and CaS receptors) homo-dimerize in their ECDs.

13.2.4 Ligand Binding Site

The discovery of GABA_B receptor heterodimerization triggered a large body of work aimed at defining the structural determinants involved in receptor activation. Similar as in other family 3 GPCRs, ligand binding occurs in the large N-terminal ECD, which is made up of two lobes (I and II) separated by a hinge region. Ligand binding modules of this kind were originally identified in bacterial periplasmic binding proteins, such as the leucine binding protein (LBP). Structural analysis proposed a model where ligand binding in a pocket made up by the two lobes results in their closure, similar to a Venus fly trap when touched by an insect. Extensive mutational analysis of the ECD of GABA_{B1} has demonstrated that residues in both lobes (S246, S269, A471, and E465 in lobe I and Y366 in lobe II) are crucial for agonist and antagonist binding. GABA and baclofen are thought to bind via their carboxylic group to the hydroxyl groups of S246 and Y366. E465 is then believed to bind to the amino terminal end of GABA. D471, which was originally proposed to undergo an ionic interaction with GABA, now appears more important for correct folding of lobe I. Mutation of S247 and Q312 increases the affinity of agonists while decreasing the affinity of antagonists. This supports a model where the LBP-like domain exists in two conformational states, an open and a closed state, where binding of ligands favors the closed state [54]. According to the three-dimensional model, a direct interaction with the second lobe is only possible in the closed form of the LBP-like domain, as shown for other receptors [55, 56]. Some mutations differentially affect the binding of agonists. For example, the potency of GABA is decreased 30-fold by the S269A mutation, whereas the potency of baclofen remains unaltered [57]. This correlates with distinct effects of Ca²⁺ on GABA and baclofen binding [57]. Interestingly, mutation of Y366 in lobe II not only decreases the affinity of GABA and baclofen but also converts baclofen into an antagonist [58]. The recently obtained crystal structure of the dimeric ECD of mGlu1 in the presence and absence of glutamate has essentially validated the homology models for GABA_B subunits described above [59]. Although both GABA_B subunits share the entire bacterial homology domain, *in silico* analysis revealed that the GABA_{B2} lacks the evolutionary constraints placed on the ligand binding pocket of GABA_{B1} or the related

mGluRs [60]. Hence, it is now generally assumed that GABA_{B2} does not bind agonist or antagonist and does not function when expressed alone.

The known activation mechanism of mGlu1 shows that binding of ligand to one of the LBP-like domains induces a conformational change bringing together both LBP-like domains with their proximal lobes. This presumably changes the conformation of the adjacent transmembrane domains, ultimately resulting in signal transduction. The available data suggest that the same mechanism is operational in the heteromeric GABA_B receptors, where binding of single GABA molecules to the LBP-like domain of GABA_{B1} is sufficient for receptor activation. In support of this, elegant experiments by Pin and colleagues demonstrated that closure of the binding domain of GABA_{B1} is sufficient for receptor activation and effector coupling [61]. Connecting the two lobes of the GABA_{B1} LBP-like domain by a disulfide bridge locks the receptor in the closed form in the active state. In agreement with this, GABA_B receptor antagonists, which bind in the open form, are no longer able to inhibit the constitutive activity of the locked receptor.

13.2.5 Molecular Determinants of G-Protein Coupling

Questions were raised as to what domains of the heteromeric GABA_{B(1,2)} complex are involved in the interaction with the G protein and whether two G proteins can bind simultaneously to the receptor. Using chimeric GABA_{B1} and GABA_{B2} subunits with swapped ECDs, it was shown that only the heptahelical region of GABA_{B2} is absolutely necessary for G-protein signaling [62, 63]. However, the heptahelical region of GABA_{B1} significantly improves coupling efficacy. It was later found that all intracellular loops of GABA_{B2} are important for receptor coupling to Kir3 channels, whereas the loops of GABA_{B1} can be replaced with those of GABA_{B2} without affecting function [64–66]. Particular attention was set on addressing the role of the second intracellular (i2) loop since there is evidence that this region is critical for G-protein coupling in family 3 GPCRs [67]. Exchanging the i2 loops between GABA_{B1} and GABA_{B2} did not result in the formation of functional receptors. Hence, the i2 loop of GABA_{B2} needs to be correctly positioned with respect to the other intracellular domains. Sequence comparison between the i2 and i3 loops of GABA_B and the related mGlu receptors highlighted clear differences between GABA_{B1} and GABA_{B2}. Mutational analysis confirmed the functional importance of conserved residues (K586, M587, and K590) in the i2 loop of GABA_{B2} and indicates that the GABA_{B1} i2 loop lacks the requirements for interaction with G proteins [64, 65]. Mutation of K686, a basic residue in the i3 loop of GABA_{B2} that plays a critical role in G-protein coupling of mGlu1 and CaS receptors [68, 69], suppresses functional coupling to G proteins in HEK293 and cerebellar granule cells, corroborating a similar role for this residue [64]. Taken together, these data illustrate the critical role of the heptahelical domain of GABA_{B2} for G-protein activation of the heteromeric GABA_B receptor. This said, one should keep in mind that all data on the importance of the GABA_{B2} subunit for G-protein coupling have been obtained in recombinant expression systems. In vivo, GABA_{B1} is clearly able to couple to G proteins and signal to effector channels in the absence of GABA_{B2} [52]. Information obtained from the crystal structure of rhodopsin and transducin suggests that only a rhodopsin homodimer provides sufficient interface to anchor both G α and G $\beta\gamma$ [70]. Although not generally accepted yet, the data on rhodopsin suggest that all

GPCRs need to homo- or heterodimerize to function. We therefore expect that the GABA_{B(1,2)} heterodimer binds one G protein only — one GABA_B subunit probably interacts with G α while the other subunit interacts with G $\beta\gamma$.

13.2.6 Allosteric Interactions Between Receptor Subunits

As described above, a significant body of work has aimed at defining the structural requirements for ligand binding, subunit interaction, and G-protein coupling. However, the interdependence of heteromerization, surface trafficking, and effector coupling makes it difficult to assign a defined molecular function to structural elements. Nevertheless a number of laboratories reached similar conclusions regarding the sequence of intra- and intermolecular events that take place when activating a GABA_B receptor [44, 62, 65, 71]. A scheme that accommodates most of the available data predicts that the ECD of GABA_{B1} is the only determinant for GABA binding, while the ECD of GABA_{B2} is necessary for receptor activation and increasing agonist affinity. The heptahelical region and the cytoplasmic tail of GABA_{B2} appears to be the prime determinant of G-protein coupling, but GABA_{B1} is clearly necessary to optimize the coupling efficiency. A model was proposed where a conformational change within the dimeric ECDs of GABA_{B1} and GABA_{B2} is responsible for the stabilization of an active dimeric form of the transmembrane domains. Hence, there is an allosteric interaction between the ligand binding domain and the effector transmembrane domains. Since GABA binding to the ECD of GABA_{B1} leads to G-protein activation by the GABA_{B2} heptahelical domain, this mechanism is also referred to as trans-activation (for a recent review see [6]). It is assumed that the GABA_B heterodimer differs from the homodimers formed by other family 3 GPCRs with respect to the functional coupling between binding and effector domains. In the GABA_B receptor the conformations of the effector and binding domains are probably tightly coupled, that is, the two domains are both in either an active or inactive conformation [71]. This model is reminiscent of a two-state model for receptor activation.

13.3 EFFECTOR SYSTEMS

13.3.1 G-Protein-Dependent and G-Protein-Independent GABA_B Effects

The best known synaptic effects of GABA_B receptors — presynaptic block of neurotransmitter release and postsynaptic hyperpolarization — are fast and mainly due to the inhibition and activation of Ca²⁺ and K⁺ channels, respectively. These effects are mediated through the $\beta\gamma$ subunits of the activated G proteins in a membrane-delimited manner not requiring any signaling through second-messenger cascades. In addition, GABA_B receptors also signal through the α subunits of the activated G proteins, which inhibits adenylate cyclase and, for example, retards the recruitment of synaptic vesicles during sustained activity and after short-term depression [72]. Moreover, GABA_B receptors regulate mitogen-activated protein kinases (MAPKs) and other cytoplasmic kinases. GABA_B signaling through second-messenger systems is generally slower and the physiological consequences are largely unknown. However, it is emerging that coupling of GABA_B receptors to intracellular

signaling cascades influences transcription, translation, stability, and activity of protein and is involved in the modulation of synaptic plasticity.

The G-protein-dependent effects of GABA_B receptor activation are largely mediated by G proteins that are members of the pertussis toxin-sensitive family G α_i /G α_o , which negatively regulate adenylate cyclase and do not couple to phospholipase C (PLC) [73, 74]. However, coupling to pertussis toxin-insensitive G proteins has been observed occasionally [75]. Moreover, recent work by Hirono and colleagues suggests that GABA_B receptors also couple to PLC in a G α_i /G α_o -linked manner resulting in the facilitation of mGlu1 responses at excitatory synapses in the cerebellum [76]. It is thought that this involves a cooperative upregulation of GPCR signaling by the G $\beta\gamma$ subunits. This is reminiscent of synergistic interactions seen with GABA_B and β -adrenergic receptors [77].

13.3.2 Ca²⁺ Channels

Presynaptic GABA_B receptors are subdivided into those that control GABA release (autoreceptors) and those that inhibit all other neurotransmitter release (heteroreceptors). In most preparations GABA_B receptors mediate their presynaptic effects through a voltage-dependent inhibition of high-voltage activated Ca²⁺ channels of the N type (Ca_v2.1) or P/Q type (Ca_v2.2/3) [78–86]. Both types of Ca²⁺ channels are expressed in presynaptic terminals and were shown to trigger neurotransmitter release [87]. A postsynaptic inhibition of Ca²⁺ channels was also postulated [88]. It was shown that GABA_B receptors couple to different types of Ca²⁺ channels depending on the input site [89]. The inhibition of Ca²⁺-inward currents is voltage dependent and varies between 10 and 42% among studies [90–92]. Since Ca²⁺ influx and transmitter release are correlated with a third- to fourth-power law [87], GABA_B agonists frequently inhibit more than 90% of neurotransmitter release with a less than 50% inhibition of Ca²⁺ channel activity. This inhibition is modulated by the action potential frequency, where strong depolarization relieves Ca²⁺ channels from their G $\beta\gamma$ -mediated inhibition [93–95]. This particular property of presynaptic Ca²⁺ channels may differentially modulate action potential trains, depending on their frequency [96]. GABA_B receptors are also described to either inhibit [81, 97, 98] or facilitate [99] L type Ca²⁺ channels. The latter effect was shown to be indirect and depend on protein kinase C (PKC) activity. Similarly, GABA_B receptors also inhibit or disinhibit T type Ca²⁺ channels [100–104]. GABA_B autoreceptors are thought to be activated only after synchronized GABA release from multiple inhibitory terminals, possibly indicating a remote localization from the synaptic cleft [105–107]. In this context it is interesting to note that it proved difficult to demonstrate presynaptic GABA_B receptors on inhibitory terminals by immunohistochemical methods, suggesting that receptor protein levels are fairly low. However, when detected, they are mostly localized to the extrasynaptic membranes [13, 108, 109]. Once activated by GABA release there is a delay of 20–50 ms prior to initiation of inhibition. Maximum inhibition then occurs after 100–200 ms and lasts up to 5 ms [110–116]. GABA_B heteroreceptors are more diverse than GABA_B autoreceptors in that they control the release of numerous neurotransmitters, including glutamate, monoamines, and many neuropeptides [1, 78–86, 117–119]. It was suggested that GABA_B heteroreceptors inhibit glutamate release via a direct inhibition of the release machinery rather than inhibiting Ca²⁺ influx [72, 120]. Heteroreceptors on

glutamatergic terminals, like GABA_B autoreceptors, are mostly localized on the extrasynaptic membrane. Activation of GABA_B heteroreceptors is thought to require a spillover of GABA from highly active GABAergic terminals. Once activated, the time course of GABA_B-induced inhibition of glutamate release is very similar to the autoreceptor-mediated inhibition of GABA.

13.3.3 K⁺ Channels

Postsynaptic GABA_B receptors induce a slow inhibitory postsynaptic current (late IPSC) through activation of inwardly rectifying K⁺ channels (GIRK or Kir3) [121, 122]. Accordingly, GABA_B-induced late IPSCs can be inhibited by the Kir3 channel blocker Ba²⁺ [123–125] and they usually exhibit a reversal potential similar to the K⁺ equilibrium potential [126, 127]. The physiological effect of Kir3 channel activation is normally a K⁺ efflux, resulting in a hyperpolarization. The time course of the late IPSC, with a time to peak of 50–250 ms and decay times of 100 and 500 ms, clearly differs from that of the fast IPSC, which is GABA_A receptor mediated [127, 128]. Baclofen-induced outward currents in hippocampal neurons are absent in *Kir3.2*, *GABA_{B1}*, and *GABA_{B2}* knockout mice, corroborating the prominent role of Kir3 channels in mediating the effects of GABA_B receptors [52, 121, 122]. Application of baclofen in the ventral tegmental area induces desensitizing and nondesensitizing Kir3 currents in dopaminergic and GABAergic neurons, respectively [129]. The difference in desensitization properties was attributed to differences in the effector Kir3 channels. The rectification properties of synaptically evoked late IPSCs differed between studies. On the one hand, the stimulus-evoked and spontaneous late IPSCs in dopaminergic neurons are inwardly rectifying and similar to those activated by baclofen [130]. On the other hand, baclofen also induces linear or even outwardly rectifying conductances, suggesting that channels other than Kir3 can contribute to the late IPSC. These other channels may include fast-inactivating, voltage-gated K⁺ channels [131] and small-conductance Ca²⁺-activated K⁺ channels (SK channels) [132]. Accordingly, the fast-inactivating A type K⁺ channel blocker 4-aminopyridine inhibits a baclofen-induced current in guinea pig hippocampal neurons [133, 134]. Moreover, GABA activates Ca²⁺-sensitive K⁺ channels [135] and small-conductance K⁺ channels [135, 136] in rat hippocampal neurons. Possibly, GABA_B receptors enhance the activity of SK channels by inhibiting the production of cyclic adenosine monophosphate (cAMP) after an action potential-induced Ca²⁺ influx [132]. Besides the well-documented coupling of GABA_B receptors to postsynaptic K⁺ channels, GABA_B receptors also appear to activate Ba²⁺-sensitive K⁺ channels at presynaptic sites [125]. Likely these presynaptic K⁺ channels are of the Kir3 type, devoid of the Kir3.2 subunit and assembled from Kir3.1 and Kir3.4 subunits [121].

13.3.4 Adenylate Cyclase

All of the known nine adenylate cyclase isoforms are expressed in neuronal tissue. Gα_i and Gα_o proteins, the predominant transducers of GABA_B receptors, inhibit most of them [137]. Many studies have reported that GABA_B receptors inhibit forskolin-stimulated cAMP formation, but others also observed a stimulation of cAMP production [138, 139]. Gα_i and Gα_o proteins inhibit adenylate cyclase types I, III, V, and VI, while Gβγ stimulates adenylate cyclase types II, IV, and VII. This

stimulation depends on the presence of $G\alpha_s$, which results from the activation of GPCRs by, for example, noradrenaline, isoprenaline, histamine, or vasoactive intestinal polypeptide [137, 140]. Therefore, the stimulatory action of $GABA_B$ receptors on cAMP levels is a consequence of G-protein crosstalk and depends on the expression of adenylate cyclase isoforms together with $GABA_B$ and $G\alpha_s$ -coupled GPCRs. Both the inhibition and enhancement of cAMP levels by $GABA_B$ receptor activation were confirmed in vivo using microdialysis [141]. Many ion channels are targets of the cAMP-dependent kinase (protein kinase A, or PKA). Accordingly, $GABA_B$ receptors can change the phosphorylation state of these proteins by influencing intracellular cAMP levels. For example, $GABA_B$ receptors increase the activity of P2X type adenosine triphosphate (ATP) receptors or the calcium-dependent K^+ current (IAHP) through a downregulation of cAMP levels, resulting in decreased PKA activity and subsequent dephosphorylation of the ion channel [132, 142]. Possibly the interplay between the GABA and ATP neurotransmitter systems could be exploited for the treatment of neuropathic pain. Significantly, the activity of $GABA_B$ receptors on adenylate cyclase is expected to modulate neuronal function on a longer time scale.

$GABA_B$ receptors were repeatedly implicated in synaptic plasticity [143–148]. Until recently it was unclear whether $GABA_B$ receptors can influence plasticity processes through the cAMP pathway. Recent experiments now demonstrate that G-protein-mediated signaling through $GABA_B$ receptors retards the recruitment of synaptic vesicles during sustained activity and after short-term depression [72]. This retardation occurs through a lowering of cAMP, which blocks the stimulatory effect of the increased Ca^{2+} concentration on vesicle recruitment. In this signaling pathway, cAMP and Ca^{2+} /calmodulin cooperate to enhance vesicle priming.

13.3.5 MAPK

MAPKs play a key role in the regulation of cellular processes. Until recently this signal transduction pathway had largely been investigated for its role in regulating cell division and differentiation in nonneuronal cells. It is emerging that MAPKs are abundantly expressed in the CNS and that in particular extracellular signal-regulated kinases (ERKs) are involved in neuronal plasticity and memory consolidation [149]. Given the role of ERK cascade in transcriptionally regulated processes, initial ERK studies focused on long-term forms of synaptic plasticity. More recent studies, however, implicate the ERKs in forms of synaptic plasticity that do not require macromolecular synthesis, such as the modulation of ion channel activity or the trafficking and insertion of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors at central synapses [150–154]. There are conflicting results on the coupling of $GABA_B$ receptors to MAPKs. It was shown that $GABA_B$ receptors can activate or inhibit ERKs [155, 156]. This discrepancy could either relate to differences in the experimental conditions or reflect that there are multiple ways for $GABA_B$ receptors to couple to MAPKs in neurons. There could be significant crosstalk between $GABA_B$ signaling pathways. For example, it is tempting to speculate that $GABA_B$ receptors couple to MAPKs through the modulation of cAMP levels and PKA. It will be interesting to see whether $GABA_B$ receptor coupling to MAPKs is important for mediating some of the $GABA_B$ effects on synaptic plasticity.

13.4 GABA_B RECEPTOR MODULATION

13.4.1 Extracellular Calcium

In addition to intra- and intermolecular interactions involved in the activation of the heteromeric GABA_B receptor, modulation can also occur via allosteric interactions with endogenous ligands as well as exogenous compounds. Investigating a possible regulation of GABA_B receptors by Ca²⁺ led to the identification of a Ca²⁺ binding site in the GABA_{B1} subunit as the first allosteric site of GABA_B receptors [57, 157]. Accordingly, it was shown that Ca²⁺ potentiates GABA-stimulated GTPγ[³⁵S] binding in membranes expressing native or recombinant GABA_B receptors. The effect of Ca²⁺ depends on the agonist, with baclofen being less sensitive than GABA or 3-aminopropyl-phosphinic acid (3-APPA). The residues that confer to GABA_B receptors the ability to sense Ca²⁺ were identified [57]. The S269A mutation in the LBP-like domain of GABA_{B1} renders the otherwise functional GABA_{B(1,2)} receptor Ca²⁺ insensitive. S269 localizes to the GABA binding pocket, next to S246 that interacts with agonists [58]. S269 in GABA_{B1} aligns with S170 in the CaS receptor, a residue that is involved in Ca²⁺ activation of the CaS receptor [158]. Allosteric regulation of GABA_{B1} by Ca²⁺ is supposed to stabilize the activated closed conformational state [57]. Possibly, Ca²⁺ compensates for the lack of the α-amino group in GABA and the contact of Ca²⁺ with S269 optimizes positioning of the carboxylic group of GABA for contacting S246. Alternatively Ca²⁺ does not directly interact with S269 but affects the positioning of its hydroxyl group. This may allow the formation of an additional hydrogen bond with the carboxylic group of GABA. The median effective concentration (EC₅₀) for Ca²⁺ modulation of GABA binding at GABA_B receptors is with 37 μM rather low. Under normal physiological conditions, with [Ca²⁺] in the blood and cerebrospinal fluid in the millimolar range, the Ca²⁺ site of GABA_B receptors is saturated. Allosteric regulation by Ca²⁺ may, however, become significant under pathological conditions, when extracellular [Ca²⁺] is low. This could be the case following ischemia [159] or epileptic seizures [160]. Recently, a GABA-independent role for the Ca²⁺-sensing property of GABA_B receptors was suggested. In cerebellar Purkinje cells, interaction of GABA_B receptors with extracellular Ca²⁺ leads to a constitutive increase in glutamate sensitivity of mGlu1 [161]. This constitutive sensitization at physiological Ca²⁺ concentrations may serve a different purpose than the transient enhancement of mGlu1 signaling by GABA_B receptor activation previously reported [76].

13.4.2 Phosphorylation and Receptor Desensitization

Modulation of GPCR activity by intracellular kinases is well known and usually the trigger for activity-dependent desensitization [162]. In general, phosphorylation of the receptor protein is followed by interaction with cytoplasmic accessory proteins called β-arrestins, which interfere with receptor–G protein coupling and promote rapid endocytosis. Both GABA_{B1} and GABA_{B2} cytoplasmic tails contain a number of consensus sites for PKC and/or PKA. Moreover, PKC is reported to suppress GABA_B-mediated inhibition of neurotransmitter release [163]. Similarly, PKA is described to desensitize GABA_B receptors expressed in *Xenopus* oocytes [164]. Subsequently, it was observed that PKC- and PKA-dependent signaling pathways

mediate the modulation of GABA_B activity in response to estrogen [165, 166]. Agonist-induced desensitization and endocytosis of heterodimeric GABA_B receptors were also observed in heterologous CHO cells [167]. However, other molecular studies contrast these findings. Moss and colleagues reported that PKA phosphorylation of S892 in the cytoplasmic tail of GABA_{B2} reduces rather than increases receptor desensitization, probably as a result of stabilizing the receptor complex at the cell surface [168]. This serine residue, at position 892 in rat and mouse GABA_{B2} is at position 893 in human GABA_{B2} due to the insertion of an additional proline residue in the signal peptide (see GenBank accession number AF099033). To simplify matters, we continued referring to it as S892 throughout the text. The data of Couve et al. challenge the conventional view that phosphorylation is a negative modulator of GPCR signaling. S892 phosphorylation in GABA_{B2} was also observed in another experimental paradigm [169]. It was shown that withdrawal from repeated cocaine treatment produces an increase in the basal level of extracellular GABA in the rat accumbens. The increase in extracellular GABA is paralleled by diminished S892 phosphorylation, suggesting a functional desensitization of GABA_B autoreceptors. At first glance contradictory, Bouvier and colleagues identified the GPCR kinase 4 (GRK4) as the kinase that promotes desensitization of GABA_B receptors [170]. Surprisingly, however, this desensitization occurred in the absence of ligand-induced receptor phosphorylation and could be promoted by GRK4 mutants deleted of their kinase domain. Again, these results are at odds with the generally accepted model linking the kinase activity of GRKs to their role in receptor desensitization. To date it is still unclear whether receptor internalization, ligand dependent or independent, is involved in the desensitization to repeated or prolonged application of GABA_B receptor agonists [171]. Recent data obtained in animal models have shown that even after prolonged baclofen application the total protein and messenger ribonucleic acid (mRNA) levels for GABA_{B1} and GABA_{B2} remained unchanged [172, 173]. It also remains to be addressed whether the clinically important development of tolerance to baclofen application is related to receptor phosphorylation or not. Identification of a potential proline-directed kinase target site in the cytoplasmic tail of GABA_{B2} (S884) by phosphoproteomic analysis of synaptosomes prepared from human cerebral cortex suggests the involvement of multiple signal transduction pathways in the regulation of GABA_B receptors [174].

13.4.3 Interacting Proteins

The discovery that receptor activity modifying proteins (RAMPs) can change the pharmacology of a GPCR triggered an intense search for proteins interacting with GABA_B receptors [175]. It was speculated that the pharmacological differences that were observed between pre- and postsynaptic GABA_B receptors relate to receptor-associated proteins that differentially interact with specific receptor populations. A number of candidate proteins were identified in yeast two-hybrid screens using the C-terminal domains of GABA_{B1} or GABA_{B2} as baits. However, there are no reports claiming pharmacological changes upon expression of these proteins together with GABA_{B1}, GABA_{B2}, or heteromeric GABA_{B(1,2)}.

Three laboratories described that the C-terminal domain of GABA_{B1} interacts with members of the ATF/CREB family of transcription factors, that is, ATF4/CREB2 and ATFx [176–178]. Gadd153, also known as CHOP, is an additional

leucine-zipper transcription factor that was described to bind to GABA_B receptors [179]. These findings are both intriguing and provocative, as they suggest a direct interaction between a GPCR and transcription factors. In addition, stimulation of GABA_B receptors resulted in transcriptional activation of ATF4/CREB2-responsive reporter genes [176, 177]. Paradoxically, in one case pertussis toxin blocked transcriptional activation, whereas in the other case pertussis toxin was ineffective. GABA_B signaling through CREB proteins was already proposed prior to cloning [180, 181]. In these earlier studies baclofen silenced transcription instead of activating it. These earlier findings are more in agreement with the well-known inhibitory effect of baclofen on cAMP production. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) genes may well be the targets of GABA_B-mediated transcriptional regulation. Both the production of NGF and BDNF are stimulated after treatment of rats with GABA_B receptor antagonists [182]. Taken together, the data suggest a novel and unique mechanism of signal transduction to the nucleus that results from activation of GABA_B receptors. Although still conflicting, the data linking GABA_B receptors to transcription factors suggest that these receptors mediate long-term metabolic effects requiring new protein synthesis. Intriguingly, a selective control of alternative GABA_{B1a} and GABA_{B1b} promoters by CREB, ATF4, and the depolarization-sensitive upstream stimulatory factor (USF) was reported [19], proposing a feedback loop regulating GABA_B isoform expression.

The intracellular C-terminus of GABA_{B1} associates with a number of proteins that are thought to regulate GABA_{B(1,2)} heteromerization and/or cell surface delivery. These include two members of the 14-3-3 family of signaling proteins [49], Marlin-1, a novel RNA binding protein [53], and msec7-1, a guanine-nucleotide exchange factor for the ARF family of GTPases [50]. Their suggested role in the assembly of functional GABA_B receptor is discussed in Section 13.2.3. The 14-3-3 proteins were implicated in GPCR signaling before [183, 184]. For example, they directly influence G-protein coupling efficiency and act as scaffolds that recruit proteins modulating G-protein activity, such as protein kinases and regulators of G-protein signaling (RGS). In addition, owing to their dimeric nature, 14-3-3 proteins might be directly implicated in the clustering of GABA_B receptors at pre- or postsynaptic sites. There are additional scaffolding proteins that interact with GABA_B receptors. *N*-Ethylmaleimide-sensitive factor, or NSF, an ATPase critical for intracellular trafficking, interacts with the C-terminus of GABA_{B2} [185]. NSF also interacts with GABA_A receptors through its binding of GABARAP [186] and possibly provides a structural link between the ionotropic and metabotropic GABA receptor systems. Tamalin is yet another scaffolding protein that interacts with GABA_B as well as mGlu receptors [187]. Tamalin is also proposed to interact with guanine-nucleotide exchange factor cytohesins and to promote intracellular trafficking and cell surface expression of associated receptors. Tamalin comprises multiple protein-interacting domains, including a 95-kDa postsynaptic density protein (PSD-95)/discs-large/ZO-1 (PDZ) domain [188], a leucine-zipper regions and a carboxy-terminal PDZ-binding motif. PDZ domain proteins are important for the scaffolding of receptors at synapses and in epithelia [189]. MUPP1, a multivalent PDZ protein, interacts with a stretch of 10 amino acids proximal to the coiled-coil domain of GABA_{B2} [4]. Interestingly this sequence does not conform to the PDZ domain binding consensus sequence. MUPP1 was initially shown to interact with another GPCR, the 5-HT_{2c} receptor [190]. MUPP1 may serve as an adaptor protein linking

GABA_{B2} to various signaling molecules. Intriguingly the GABA_{B1} C-terminus contains a putative PDZ domain binding consensus sequence, LYK. However, no PDZ domain protein is reported to interact with GABA_{B1}. Finally, the actin binding protein β -filamin is proposed to tether GABA_B receptors via the GABA_{B2} subunit to the cytoskeleton [185].

Two reports suggest that GABA_B receptor ECDs interact with other proteins. Fibulin, an extracellular matrix protein, apparently binds to the sushi repeats of the GABA_{B1a} subunit [20]. This finding is of special interest, as we still do not know whether mechanisms are in place to compartmentalize GABA_{B1a} and GABA_{B1b} to different subcellular regions, for example, pre- and postsynaptic sites. The other report provides evidence that the HNK-1 carbohydrate carried by many neural extracellular matrix proteins, among them tenascin-R and tenascin-C, binds to GABA_B receptors [191]. HNK-1 was proposed to regulate GABA_A receptor-mediated perisomatic inhibition by suppression of postsynaptic GABA_B receptor activity. HNK-1 does not bind to the sushi repeats of GABA_{B1a}.

GABA_B receptors were found to be associated with lipid rafts, specialized plasma membrane microdomains that function as platforms for signaling complexes [192]. Lipid rafts are enriched in cholesterol/sphingolipids and contain specific populations of membrane-associated proteins, such as G-proteins and other signaling molecules. For example, G α_i - and G α_o -type G proteins, the main transducers of GABA_B receptors, are enriched in the lipid-raft fraction extracted from cerebellar membranes. It remains to be seen whether lipid rafts can segregate GABA_B receptor populations (e.g., GABA_{B(1a,2)}} and GABA_{B(1b,2)}}), and in some ways account for the functional heterogeneity observed in vivo.

13.5 GABA_B-DEFICIENT MICE

Given that cloning efforts did not substantiate the claim for receptor heterogeneity, it became important to understand to which GABA_B functions the cloned receptors can contribute in vivo. To address this question, a number of laboratories generated knockout mice lacking either GABA_{B1} [122, 193, 194] or GABA_{B2} [52, 195]. Only the GABA_B knockout mice generated on the Balb/c genetic background are viable [52, 122]. The GABA_B knockout mice generated on other genetic backgrounds die within three to four weeks after birth, thus precluding behavioral analysis of adult animals [193–195]. Strain differences in viability of knockout mice are not uncommon [196]. The overt phenotype of all GABA_B-deficient mice includes spontaneous epileptic seizures and these seizures may be suppressed to some extent in the Balb/c genetic background. A reduced seizure activity, in turn, may rescue mice from lethality. Analysis of GABA_{B1}^{-/-} and GABA_{B2}^{-/-} mice revealed that deletion of either subunit is sufficient to abolish all well-known GABA_B responses. Upon GABA_B agonist application, GABA_{B1}^{-/-} and GABA_{B2}^{-/-} mice show neither the typical muscle relaxation and hypothermia nor delta EEG waves. These behavioral findings are paralleled by a loss of all detectable biochemical and typical pre- and post-synaptic electrophysiological GABA_B responses. However, whereas GABA_B-mediated responses are completely lacking in GABA_{B1} knockout mice, an atypical response is observed in GABA_{B2}-deficient mice [52]. In CA1 hippocampal neurons baclofen inhibits instead of activates K⁺ channels. Whether a physiological relevant

signaling underlies these atypical GABA_B responses in GABA_{B2}^{-/-} mice is unclear. It is possible that these responses are a consequence of the knockout situation where GABA_{B1} is expressed in the absence of its usual dimerization partner. These findings substantiate that all classical GABA_B responses in vivo relate to heteromeric GABA_{B(1,2)} receptors. The heteromeric nature of predominant native GABA_B receptors is further emphasized by the substantial down regulation of GABA_{B2} protein in GABA_{B1}^{-/-} mice and vice versa. In conclusion, knockout studies demonstrate that GABA_{B1} as well as GABA_{B2} are essential components of abundant pre- and postsynaptic GABA_B receptors in the CNS. Analysis of GABA_{B1}^{-/-} mice further indicates that the GABA_{B1} subunit is an essential requirement for GABA_B receptor function in the peripheral nervous system and the enteric nervous system [197]. Therefore, gene-targeting experiments do not substantiate the existence of GABA_B subtypes in the periphery, claimed by studies in which different rank orders of GABA_B agonist affinities were reported.

Theoretically, it remains possible that additional GABA_B subunits exist, which could explain the discrepancy between the historically diverse receptor pharmacology in vivo on the one hand and the lack of pharmacologically distinct cloned receptors on the other hand. However, with most of the human genome sequence available, and despite extensive data-mining efforts, no GABA_B genes other than *GABA_{B1}* and *GABA_{B2}* were identified. All GABA_B-related proteins that were isolated do not form functional GABA_B receptors when expressed together with GABA_{B1} and/or GABA_{B2} [198–203]. Altogether, this indicates that the likelihood of identifying additional GABA_B receptor genes in the future is small.

13.6 PHARMACOLOGY

13.6.1 Endogenous GABA_B Ligands: GABA and GHB

Over the past decade it was emerging that GABA is not the only endogenous GABA_B ligand. It is now generally accepted that GHB, a metabolite of GABA that is present at micromolar concentration in the brain, interacts with GABA_B receptors [156, 204–206]. Patients suffering from GHB aciduria, a congenital enzyme defect causing a GHB accumulation, exhibit psychomotor retardation, delayed or absent speech, hypotonia, ataxia, hyporeflexia, seizures, and EEG abnormalities. GHB binds to native and recombinant GABA_B receptors, however with significantly lower affinity than to its cognate high-affinity [³H]GHB binding sites [206]. Therefore, GHB is only expected to activate GABA_B receptors when endogenous GHB levels increase above physiological levels, due either to a genetic disease or to exogenous administration of GHB (see below). The physiological role of endogenous GHB is unclear. However, if endogenous GHB is involved in active signaling, high-affinity GHB receptors, likely related to brain [³H]GHB binding sites, are expected to mediate these effects. The availability of GABA_B-deficient mice provided the opportunity to study GHB effects in the absence of coincident GABA_B effects [194, 207, 208]. Following GHB application, GABA_{B1}^{-/-} mice did not show the typical hypolocomotion, hypothermia, increase in striatal dopamine synthesis, or EEG delta-wave induction seen in wild-type mice. This indicates that these GHB effects are all mediated by GABA_B receptors. Autoradiography reveals a similar spatial

distribution of [^3H]GHB binding sites in brains of $\text{GABA}_{\text{B1}}^{-/-}$ and wild-type mice, demonstrating that GABA_{B} subunits are not part of high-affinity [^3H]GHB binding sites. Millimolar concentrations of GHB induce small $\text{GTP}\gamma[^{35}\text{S}]$ responses in brain membrane preparations from wild-type but not from $\text{GABA}_{\text{B1}}^{-/-}$ mice. The $\text{GTP}\gamma[^{35}\text{S}]$ responses in wild-type mice are blocked by the GABA_{B} antagonist CGP54626, but not by the GHB antagonist NCS-382. This, together with additional data [209], suggests that the GHB-induced $\text{GTP}\gamma[^{35}\text{S}]$ responses are exclusively mediated by GABA_{B} receptors and not by the high-affinity [^3H]GHB binding sites, as proposed in earlier studies [210, 211].

Recently, the cloning of a putative GHB receptor from a rat hippocampal cDNA library was reported [212]. However, several findings related to this putative GHB receptor require clarification. For example, the N-terminal part of the cloned GHB receptor identifies it as a member of the tetraspannin gene family, with four transmembrane regions and no significant similarity to GPCRs. Nevertheless, the cloned protein is reported to exhibit seven transmembrane regions and to activate G proteins. No additional transmembrane domains to achieve the typical GPCR topology are visible in the C-terminal part of the protein, which exhibits no significant homology to known proteins. All the more puzzling, the mRNA of the putative GHB receptor is particularly abundant in the cerebellum, where GHB binding sites are low or absent [204]. Furthermore, the GHB receptor antagonist NCS-382 has no activity at the cloned GHB protein. In summary, these findings make it unlikely that the cloned protein corresponds to the high-affinity [^3H]GHB binding sites in the brain.

In the past GHB was clinically used as an anesthetic, while in recent times it is shown to normalize sleep patterns in narcoleptic patients [213, 214]. GHB (Xyrem) is used to reduce the number of cataplexy attacks in patients with narcolepsy [215]. Preliminary preclinical and clinical data suggest that GHB is also useful in the therapy of alcoholism, nicotine, and opiate dependency [216], similar to baclofen. However, in contrast to baclofen, GHB is recognized as a drug with a strong abuse potential itself [214] (see Section 13.7.2).

13.6.2 Agonists and Competitive Antagonists

13.6.2.1 Agonists. The prototypic GABA_{B} agonist β -[4 chlorophenyl] GABA (baclofen, Lioresal) was synthesized in 1962 and introduced to the market as an antispastic agent in 1972 [217]. Thus baclofen was in clinical use long before GABA_{B} receptors were identified as a distinct entity [1]. Since then 3-APPA (CGP27492) and its methyl homolog (3-APMPA, CGP35024, identical with SK&F 97541) have emerged and are reported to be three-to seven fold more potent than the active isomer of baclofen. The latter compounds are also available as radioligands. Other methyl phosphinic acid-based agonists have been produced, such as CGP44532 and its (R)-(+)-enantiomer CGP44533. Interestingly, ethyl (and higher homolog) phosphinic acid derivatives (e.g., CGP36216) are GABA_{B} antagonists [218]. The difluoromethyl phosphinic acid derivative CGP47656, with a substituent the size of which is between a methyl and an ethyl group, showed properties of a partial GABA_{B} agonist [219]. GABA_{B} receptor agonists display a great number of pharmacological effects, including central muscle relaxation, antinociception, epileptogenesis, and suppression of cocaine and nicotine/opioid self-administration. As mentioned above, GHB (Xyrem) has been recently introduced to the market [215]. GHB is a partial

agonist at GABA_B receptors [206]. Since no physiological responses of GHB are observed in GABA_B knockout mice [194, 207], it is likely that the therapeutic effects of Xyrem are due to activation of GABA_B receptors.

13.6.2.2 Antagonists. Kerr and colleagues synthesized the first GABA_B antagonists, phaclofen and 2-hydroxy saclofen [220, 221]. Both compounds exhibit only low micromolar affinities for brain GABA_B sites but were instrumental in advancing our understanding of GABA_B receptor function. Subsequently, the first antagonists that cross the blood–brain barrier after intraperitoneal (CGP35348) or oral administration (CGP36742) were synthesized [144, 147]. Bolser and colleagues reported SCH50911, a structurally distinct GABA_B antagonist that also acts centrally after peripheral administration [222]. Nonetheless all these later compounds suffered from low potency. A breakthrough was the development of the phosphinic acid derivatives CGP52432, CGP55845A, CGP54626A, CGP56433A, CGP61334, and CGP62349. These antagonists now reached nanomolar affinities at GABA_B receptors [218, 223]. Subsequently several high-affinity radioligands were developed which ultimately permitted expression cloning of the first GABA_B receptor cDNA [3] (see Section 13.2.1). Like GABA_B agonists, GABA_B antagonists exert a great number of physiological effects. For instance, they suppress absence seizures in various rodent models or improve cognitive performance in a variety of learning paradigms (see Section 13.7).

Using the above-described GABA_B compounds numerous biochemical and physiological studies postulate pharmacological differences between auto- and heteroreceptors and even within auto- and heteroreceptors [119, 224–228]. However, the proposal of presynaptic receptor subtypes based on neurotransmitter release experiments has been open to dispute [229]. Electrophysiological and release experiments suggest distinctions between pre- and postsynaptic GABA_B receptors as well [125, 230–238]. Accordingly, published half-maximal effective concentrations for baclofen in pharmacological studies vary considerably and range between 500 nM and 15 μ M.

The rank order of agonist and antagonist binding affinities at GABA_{B1} and native GABA_B receptors is identical [3]. This, together with the reasons outlined in Sections 13.2.2, 13.5, and 13.6.3.2, makes it unlikely that molecularly distinct GABA_B receptor subtypes or isoforms underlie these pharmacological differences. Obviously, the ratio of receptors and effectors can determine the apparent potency of receptor agonists [239]. Agonist potency may also depend on the concentration of divalent cations in the extracellular buffer, the association with lipid rafts [192], the phosphorylation state of subunits, or the type of G protein that is present in the cell. These factors may also explain why the agonist affinity at GABA_B receptors increases 10-fold during postnatal development [22]. This said, all experiments with native GABA_B receptors reporting changes in the rank order of ligand efficacies remain unexplained [224, 231]. These experiments would normally clearly argue in favor of pharmacologically distinct receptor subtypes.

13.6.3 Novel GABA_B Compounds

13.6.3.1 Allosteric Modulators. The side effects of baclofen, principally sedation, tolerance, and motor impairment, limit its utility for the treatment of many diseases.

Novel GABA_B drugs that largely lack these components are therefore much sought after. In the absence of pharmacological subtypes, alternative strategies for achieving selectivity must be considered. For instance, positive allosteric modulators may provide a means to dissociate the unwanted side effects seen with baclofen. Allosteric modulators discriminate between activated and nonactivated receptor states. They will enhance the endogenous activity of GABA, in contrast to agonists that will activate every GABA_B receptor they reach, independently of synaptic activity. To be effective, allosteric GABA_B drugs therefore rely on receptor activity stimulated by endogenous GABA. The demonstration of an intrinsic brake on memory impairment, locomotion, bladder activity, and nociception in GABA_{B1}^{-/-} and GABA_{B2}^{-/-} mice [52, 122, 197] is therefore of great importance (Section 13.5). This suggests that under physiological conditions disease-relevant neuronal systems are under phasic or tonic control of GABA_B receptors and that a treatment with allosteric modulators is possible.

Using cloned GABA_B receptors, several laboratories developed functional assay systems. For instance, the activation of G proteins by GABA_{B(1,2)} can be measured using the GTPγ[³⁵S] binding assay [240]. Alternatively mobilization of intracellular Ca²⁺ can be detected in cell lines expressing GABA_{B(1,2)} in combination with chimeric G proteins [41]. Functional assay systems permitted the identification of the first synthetic allosteric GABA_B compounds [240, 241]. CGP7930, CGP13501, GS39783 and related allosteric compounds are structurally distinct from GABA_B agonists and markedly enhance agonist-stimulated responses at GABA_B receptors (Fig. 13.3). Notably, these allosteric compounds have little or no intrinsic activity and do not directly activate GABA_B receptors [242]. Where analyzed, GABA concentration–response curves in the presence of fixed concentrations of the allosteric modulator indicate an increase of both the potency and the maximum efficacy of GABA at GABA_{B(1,2)} receptors. The published allosteric compounds do not discriminate between GABA_{B(1a,2)} and GABA_{B(1b,2)}, the two predominant GABA_B receptor populations in the nervous system. The precise binding sites for allosteric modulators are unknown, but all compounds require the presence of GABA_{B2} to exert their allosteric effect. Possibly, the compounds bind to the GABA_{B2} subunit itself or to the GABA_{B1}/GABA_{B2} interface. Indeed, using interspecies rat/*Drosophila* subunit combinations, it was recently shown that the positive modulator GS39783 binds to the GABA_{B2} subunit [243]. Moreover, it is suggested that CGP7930 directly

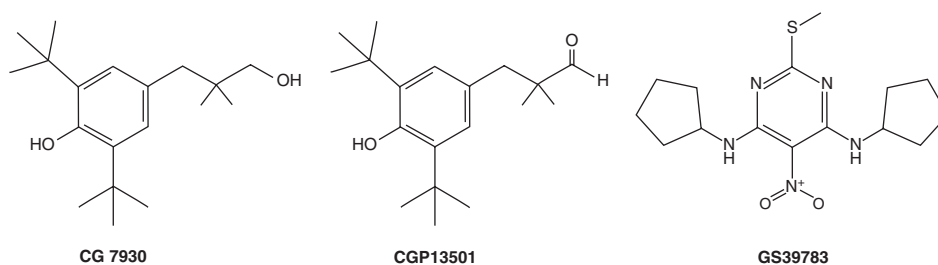


Figure 13.3 Positive allosteric modulators acting at GABAB receptors. Chemical structures described by Urwyler et al. [240, 241]. CGP7930 [2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethylpropyl)-phenol], its aldehyde analog CGP13501, and GS39783 [*N,N'*-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine] are shown.

acts on the transmembrane domain of GABA_{B2} [244]. Therefore it is conceivable that all these hydrophobic compounds interact with the transmembrane domains, similar to allosteric modulators at mGlu receptors (see below). Kerr and colleagues [245] described the arylalkylamines as a different class of positive allosteric GABA_B modulators. However, this is not supported by subsequent experiments in another laboratory [246].

Positive allosteric modulation is not the sole possibility to fine tune GABA_B receptors. High-throughput screening using functional read-outs allows identification of noncompetitive GABA_B antagonists as well. This has been demonstrated for the structurally related mGlu receptors [247]. Noncompetitive mGlu antagonists, such as CPCCOEt, MPEP, and BAY36-7620, decrease the maximal effect of glutamate without changing its affinity, clearly indicating that they interact at a site other than the glutamate binding site. Similar to positive allosteric modulators, noncompetitive mGlu receptor antagonists interact with the transmembrane domains [248–250]. Noncompetitive GABA_B antagonists, based on novel chemical structures, have so far not been disclosed.

13.6.3.2 Subtype-Selective Ligands. To date the only prominent molecular distinction in the GABA_B system is based on the two splice variants GABA_{B1a} and GABA_{B1b}. Essentially, these two variants represent the only means for directing the search for novel GABA_B drugs toward molecularly distinct receptor populations. The differential expression patterns of GABA_{B1a} and GABA_{B1b} point to a functional heterogeneity, but it is unknown which physiological effects relate to which isoform. Most importantly, it remains unclear which splice variants are involved in pre- and postsynaptic functions [23, 25, 28, 29, 251–253].

Appreciating the unique roles played by GABA_{B1a} and GABA_{B1b} is essential to fully exploit GABA_B receptors for therapeutic uses. It was argued that the anticonvulsant, antihyperalgesic, and anxiolytic drug gabapentin is able to distinguish the two variants [33, 34]. In particular, gabapentin was claimed to be active at GABA_{B(1a,2)} but not at GABA_{B(1b,2)} receptors. However, in most people's hands gabapentin has no activity at GABA_B receptors [37, 38, 254, 255]. The reason for this discrepancy is unclear. Given the lack of truly selective ligands, it will probably be necessary to take a genetic approach to dissociate in vivo functions of GABA_{B(1a,2)} and GABA_{B(1b,2)} receptors. Mice with selective ablations of the GABA_{B1a} or GABA_{B1b} subunits should allow exposing the influence of splice variants on the manifestation of physiological and behavioral traits. Such mice will also help to evaluate whether compounds specifically targeted to GABA_{B(1a,2)} or GABA_{B(1b,2)} are likely to have distinct enough effects to warrant drug discovery efforts.

13.7 DISEASE

13.7.1 GABA_B Receptors as Therapeutic Targets

GABA_B receptors have been implicated in a wide variety of neurological and psychiatric disorders. To date, two GABA_B drugs are on the market, baclofen (Lioresal) and GHB (Xyrem). Lioresal is used to treat severe spasticity of cerebral and spinal origin. Baclofen is effective in animal models for many central and

peripheral disorders, but side effects and tolerance prohibited a more widespread use of this drug in humans. GHB (Xyrem) has been approved in different countries for general anaesthesia and the treatment of alcohol withdrawal and addiction. In the United States, GHB (Xyrem) is a controlled substance and only approved by the Food and Drug Administration for treating a small population of patients with narcolepsy who experience episodes of cataplexy. It was also marketed as a body-building and fat-burning compound and is widely used and abused as a recreational drug due to the euphoria it produces (for a recent review see [214]). Similarly, GABA_B antagonists show therapeutic promise but their shortcomings (e.g., lack of brain penetration or some proconvulsive potential) hampered clinical development. Nevertheless, the low-affinity compound SGS742 (CGP36742) has recently entered clinical trials in patients with mild cognitive impairment (MCI) [256]. Gene knockout mice support the preclinical and clinical evidence that GABA_B drugs could be used to manage pain, strengthen memory, and treat epilepsy [52, 122, 193]. Moreover, GABA_{B1}^{-/-} and GABA_{B2}^{-/-} mice point at indications that were not necessarily linked to the GABA_B system. For example, these mice exhibit a hyperactive phenotype reminiscent of dopamine transporter DAT knockout mice that are similarly aroused by novelty and respond with hyperlocomotion to a new environment [52, 122, 257]. It is commonly assumed that hyperactive behaviors are related to a hyperdopaminergic state. It is therefore conceivable that the loss of GABA_B control over dopamine release triggers the behavioral abnormalities in GABA_{B1}^{-/-} and GABA_{B2}^{-/-} mice. A GABA_B brake on dopamine release could possibly be exploited to attenuate motor problems of Parkinson's disease or attention-deficit hyperactivity disorder (ADHD) patients. Furthermore, GABA_{B1}^{+/-} mice show enhanced prepulse inhibition when compared to wild-type littermates, suggesting that GABA_{B1} knockout mice exhibit sensorimotor gating abnormalities [193]. Compounds interfering with GABA_B receptors may therefore be beneficial in schizophrenia. Below we discuss some of the most promising indications for GABA_B drugs in more detail.

13.7.2 Addiction

There is now good evidence that GABA_B agonists can reduce the craving for drugs such as cocaine, heroin, alcohol, and nicotine [258]. Preliminary clinical studies with cocaine-abusing patients reported a reduced craving for cocaine following baclofen administration [259, 260]. GABA_B agonists are also effective in clinical studies of alcohol abuse [261]. Studies in animals generally support that baclofen and other compounds acting at GABA_B receptors have a therapeutic effect on abuse of a host of addictive substances (for a recent review see [262]). There is good evidence that this is due to an attenuation of the reinforcing effects of abused drugs in the mesolimbic dopamine system [263, 264]. Drugs of abuse increase extracellular dopamine levels in the accumbens, a brain region that is believed to be involved in the reward and reinforcement circuitry [265, 266]. Activation of GABA_B receptors in the accumbens reduces firing of dopaminergic cells and inhibits the release of dopamine [267]. Altogether, it appears that GABA_B activity blocks the increase in dopamine release that is otherwise induced by drugs of abuse [268]. Accordingly, rats reduce self-administration of cocaine after they receive an injection of baclofen into the accumbens and the ventral tegmental area [269, 270]. Interestingly, different GABA_B

agonist can exert opposing behavioral effects on the reward pathway. Whereas baclofen reduces self-administration and reinstatement of a number of drugs in rodents, GHB is readily self-administered in mice [271] and has a strong abuse potential itself [214]. A recent study by Lüscher and colleagues [129] offered a pharmacological explanation for the difference in abuse liability of GHB and baclofen. A different coupling efficacy of GABA_B receptors to GIRK (Kir3) channels in dopaminergic and GABAergic neurons of the ventral tegmental area may explain bidirectional effects of GABA_B agonists on the mesolimbic dopamine system [129]. Nevertheless, GHB also showed promising effects in situations involving opiate withdrawal and alcohol and nicotine dependence [216] and was shown to decrease cocaine self-administration in rats [272]. Withdrawal from repeated cocaine treatment produces an increase in the basal levels of extracellular GABA in the nucleus accumbens. This results in functional desensitization of GABA_B receptors concomitant with a diminished S892 phosphorylation of the GABA_{B2} subunit [169]. It remains to be addressed whether this functional desensitization of GABA_B receptor after drug withdrawal restricts the therapeutic potential of GABA_B receptor agonists. In conclusion, preclinical and clinical data support that modulation of GABA_B receptors could be used to treat addiction. Positive modulators of GABA_B receptors could be favorable for therapy and avoid some of the side effects of full agonists [273].

13.7.3 Anxiety and Depression

It was proposed many years ago that pharmacological intervention with GABA_B receptors could be effective in the treatment of mood disorders such as anxiety and depression. Chronic exposure to antidepressant drugs was shown to upregulate GABA_B binding sites in the rat frontal cortex [274]. It appears that a decrease in GABA_B-mediated neurotransmission is compensated for by an increase in post-synaptic GABA_B receptor protein. These findings were extended by showing that baclofen inhibits the action of antidepressants [275, 276]. In line with this the antagonist CGP36742 exhibited antidepressive properties in the learned helplessness paradigm in rats [277]. Accordingly, it was proposed that GABA_B antagonists could be effective in the treatment of depression. However, a positive modulation of GABA_B receptors as an antidepressant therapy was also suggested [278].

Similarly, pharmacological intervention with GABA_B receptors was also investigated as a treatment of anxiety disorders. Baclofen showed anxiolytic-like effects in several preclinical tests in rodents [279–282], and reverses the anxiogenic response induced by withdrawal from chronic diazepam and alcohol treatment [283–285]. Moreover, baclofen attenuates the anxiety associated with alcohol withdrawal [286], post-traumatic stress [287], panic disorder [288], and traumatic spinal cord lesions in the clinic [289].

Recently the development of novel pharmacological and genetic tools advanced the knowledge on the role of GABA_B receptors in emotional disorders (for a recent review see [290]). Mice lacking GABA_B receptors show altered depression and anxiety-related behavior in a variety of experimental paradigms [291, 292]. In particular, GABA_{B1}^{-/-} mice display antidepressant-like activity in the forced-swim test, in support of the antidepressant action of GABA_B antagonists. GABA_{B1}^{-/-} as well as GABA_{B2}^{-/-} mice are more anxious than their wild-type counterparts. Moreover, GABA_{B1}^{-/-} mice have a paniclike response in the elevated

zero maze [291]. These data suggest that activation of GABA_B receptors might be anxiolytic. Recent studies with positive modulators of GABA_B receptors (i.e., CGP39783) indeed indicate that such compounds might represent a novel class of anxiolytic with a superior side-effect profile than currently available medication [291, 293]. Taken together, targeting GABA_B receptors with allosteric compounds might provide a useful strategy in the treatment of mood disorders. However, how activation of GABA_B receptors affects depression and anxiety is not understood yet.

13.7.4 Epilepsy

GABA_B receptors have repeatedly been implicated in the etiology of epilepsies. However, the first genetic link between GABA_B receptors and human epilepsy was only provided very recently [294]. It appears that a *GABAB1* polymorphism not only confers a highly increased susceptibility to temporal lobe epilepsy but also influences the severity of the disease.

GABA_B antagonists suppress the absence seizures seen in the *lh/lh* lethargic mice [295] and the genetic absence-epilepsy rats from Strasbourg (GAERS) [296], while agonists exacerbate the seizures. Apparently, blocking of thalamic GABA_B receptors reverses an excess of inhibition that is the cause of absence seizures [100, 297]. It was proposed that GABA_B-mediated IPSPs have a “priming” function toward the generation of low-threshold Ca²⁺ potentials, thereby facilitating burstfiring of the type observed in absence epilepsy [100]. The primary gene defect in *lh/lh* mice is a mutation in the Ca²⁺ channel β subunit, which affects the interaction with the G $\beta\gamma$ subunits of the activated G protein. The phenotype of *lh/lh* mice involves an upregulation of GABA_B binding sites, which is expected to facilitate absence seizure development. Paradoxically, the overt phenotype of GABA_B receptor-deficient mice includes spontaneous seizures, including sporadic absence-type seizures [52, 122, 193, 298]. However, the absence-type seizures seen in the GABA_{B1}^{-/-} mice are not directly comparable to the “typical” absence seizures observed in the GAERS. The seizures in the GAERS are characterized by frequent and short EEG bursts, while the ones seen in the GABA_{B1}^{-/-} mice are rare and of much longer duration. A lack of GABA_B signaling may also underlie the clonic-type seizures in the *weaver* mouse [299]. The *weaver* mouse exhibits a mutation in the *Kir3.2* K⁺-channel gene, which leads to a postsynaptic loss of GABA_B inhibition and to seizure development [121]. Kainic acid-induced seizures in rats are characterized by a downregulation of GABA_B receptor subunits [300, 301]. In contrast, following induction of generalized seizures in rats with electroshock, an upregulation of the GABA_{B1b} mRNA, but not of the GABA_{B1a} mRNA, was observed [302]. This suggests a selective involvement of GABA_B isoforms in the regulation of electroshock-induced seizure activity.

To date, the data indicate that GABA_B compounds could be most useful in the pharmacotherapy of absence seizures in children. The finding, however, that high doses of certain GABA_B antagonists induce convulsions in rats renders clinical trials problematical [303]. Clearly, a wide therapeutic window will be a critical requirement when entering clinical trials with GABA_B antagonists.

13.7.5 Nociception

Baclofen exerted antinociceptive effects in clinical trials involving trigeminal, glossopharyngeal, vagoglossopharyngeal and ophthalmic-postherpetic neuralgias,

diabetic neuropathy, and migraine [101, 304–306]. Although baclofen is used clinically to treat neuropathic pain and, when administered intrathecally, to attenuate pain associated with spinal injury [307] or stroke [308], its use as a general analgesic is limited because of its sedative properties and the rapid development of tolerance to its pain-relieving activity. Baclofen also exhibited antinociceptive properties in rodent models of acute pain, such as the tail-flick, acetic acid writhing, formalin, and hot-plate tests [309, 310]. Moreover, baclofen showed antinociceptive and antiallodynic actions in chronic pain models in rats [311, 312]. Baclofen likely exerts its effects in both the spinal cord [313] and brain [314, 315]. It has been suggested that an inhibitory tone exerted via GABA_B receptors can control primary afferent-mediated activation of dorsal horn projection neurons in rats and monkeys [316, 317]. Recently, a study by Nagy and colleagues provided an alternative mechanism [318]. They showed that GABA_B receptors exert a direct control on dorsal horn neuron excitability in rat spinal cord slice preparation. One of the few cortical areas consistently activated by painful stimuli is the rostral agranular insular cortex (RAIC) where GABA robustly inhibits neuronal activity [314]. Selective activation of GABA_B receptor-bearing RAIC neurons produces hyperalgesia through projections to the amygdala, an area involved in the control of pain and fear. In the dorsal horn of the spinal cord, baclofen inhibits the release of glutamate and substance P from both large and small fibers [39]. This explains why intrathecal application of baclofen relieves central pain in patients with spinal lesions [307] or after cerebral strokes [308]. Acute pain tests with GABA_B receptor-deficient mice support that GABA_B receptors participate in nociceptive pathways [52, 122]. GABA_{B1}^{-/-} as well as GABA_{B2}^{-/-} mice exhibit pronounced hyperalgesia to noxious heat in the hot-plate and tail-flick tests as well as reduced paw withdrawal thresholds to mechanical pressure. Changes in the noxious thermal and mechanical threshold suggest that there is a loss of intrinsic GABA_B tone in the nociceptive system of GABA_B-deficient mice. The lack of GABA_B receptors most probably results in an increased central hyperexcitability of the spinal nociceptive pathways.

13.7.6 Tumor Cell Growth and Migration

An emerging topic is the involvement of GABA_B receptor signaling in the control of tumor cell growth and migration. It is suggested that GABA_B receptors control the migration and vectorial growth of cells in the nervous system [319–322]. Masaharu Tatsuta and colleagues were the first to show that systemic administration of baclofen significantly reduces the incidence and number of gastric cancers in rats and attenuates azoxymethane-induced carcinogenesis in rat colon [323, 324]. Similarly, baclofen completely inhibited the noradrenaline-induced migration of SW480 colon carcinoma cells [325], supporting that baclofen or the newly available allosteric modulators could be used to prevent cancer cell migration.

13.7.7 Genetic Linkage Studies

The *GABA_{B1}* and *GABA_{B2}* genes map to human chromosomes 6p21.3 and 9q22, respectively [326, 327]. Given the potential implication of GABA_B receptors in the etiology of epilepsies (Section 13.7.4) a number of studies addressed a direct involvement of the *GABA_{B1}* gene. The result of a recent study indicates that a

GABA_{BI} polymorphism, G1465A, indeed confers a highly increased susceptibility to temporal lobe epilepsy [294]. This polymorphism also influences the severity of this common epileptic disease. This agrees with earlier studies that proposed a dysfunctional *GABA_B* system as one of the causes of temporal lobe epilepsy [301, 328, 329]. A possible involvement of *GABA_{BI}* receptors in idiopathic generalized epilepsies was investigated in linkage and association studies [330, 331]. Yet another study explored a link to childhood absence epilepsy [332]. However, these latter studies provided no evidence for an involvement of *GABA_{BI}* polymorphisms in these two other forms of epilepsy. Similarly, Anaya and colleagues found no linkage between the *GABA_{BI}* gene and epilepsy in Caucasian patients [333]. Other linkage studies addressed a possible involvement of the *GABA_{BI}* gene in conditions of alcohol dependence [331] and in panic disorders [334]. However, *GABA_{BI}* polymorphisms do not account for the genetic variance of alcohol dependence, nor is there an indication for increased vulnerability to panic disorders. The *GABA_{B2}* gene also maps to a number of disease loci. Most importantly, *GABA_{B2}* is located within a region of chromosome 9q12 that showed a linkage to nicotine dependence [335]. A recent study provided evidence of a significant association of *GABA_{B2}* variants with nicotine dependence, implying that this gene plays a role in nicotine addiction [336]. *GABA_{B2}* also maps in the vicinity to the locus for hereditary sensory neuropathy type 1 (HSN-1) [327]. However, a possible involvement of *GABA_{B2}* in the etiology of HSN-1 has not yet been investigated.

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14

VOLTAGE-GATED ION CHANNELS

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14.1	Introduction	618
14.1.1	Cloning and Evolutionary Relationships	619
14.1.2	Nomenclature	621
14.2	Potassium Channels	621
14.2.1	Voltage-Gated K ⁺ Channels	623
14.2.2	Selectivity	623
14.2.3	Voltage Activation	625
14.2.4	Gating	626
14.2.5	Fast Inactivation	627
14.2.6	Interacting Proteins	627
14.2.7	Physiological Functions, Disease Relevance, and Pharmacology	628
14.2.7.1	K _v Channels	628
14.2.7.2	Large-Conductance Calcium-Activated K ⁺ Channels	630
14.2.7.3	Intermediate-Conductance Calcium-Activated K ⁺ Channels	631
14.2.7.4	Small-Conductance Calcium-Activated K ⁺ Channels	631
14.2.7.5	KCNQ Channels	632
14.2.7.6	KCNH K ⁺ Channels	633
14.2.8	Other Subfamilies of K ⁺ Channels: Inwardly Rectifying and Two-P K ⁺ Channels	634
14.3	Voltage-Gated Sodium Channels	635
14.3.1	Single Family of Voltage-Gated Na ⁺ Channel α Subunits	636
14.3.2	Selectivity	637
14.3.3	Inactivation	637
14.3.4	Trafficking	638
14.3.5	Physiological Functions	639
14.3.6	Channelopathies	641
14.3.7	Pharmacology	642
14.4	Voltage-Gated Calcium Channels	643
14.4.1	α Subunit	644
14.4.2	β Subunits	645
14.4.3	$\alpha_2\delta$ Subunits	646

14.4.4	γ Subunits	646
14.4.5	Function and Pharmacology	646
14.4.5.1	General Blockers	647
14.4.5.2	Ca_v1 Family	647
14.4.5.3	Ca_v2 Family	648
14.4.5.4	Ca_v3 Family	649
14.4.5.5	Auxiliary Subunit Modulators	650
14.5	Other Voltage-Gated Channels	650
	References	651

14.1 INTRODUCTION

Cells create differences in voltage and ion concentration across membranes through the action of adenosine 5'-triphosphate (ATP)-dependent ion pumps, and the dissipation of these electrochemical gradients through ion channels underlies the generation and propagation of action potentials, release of hormones and neurotransmitters, and many other functions essential for cellular signaling and homeostasis. Mammalian cell membranes typically maintain a resting membrane potential of -50 to -90 mV, a range that is close to the equilibrium potential for potassium (K^+) and far from the equilibrium potentials for sodium (Na^+) and calcium (Ca^{2+}). Relatively voltage-insensitive background K^+ channels maintain hyperpolarized membrane potentials at rest, resisting the depolarizing shifts in membrane potential that open voltage-dependent Na^+ , K^+ , and Ca^{2+} channels.

A sufficiently strong depolarizing stimulus, such as a sodium current through an ionotropic glutamate receptor, can precipitate an action potential by initiating the sequential opening and closing of voltage-gated ion channels. Voltage-gated Na^+ channels open earliest and further depolarize the membrane before they inactivate and become nonconductive. More slowly activating voltage-gated K^+ channels then open and return the membrane toward its resting potential before they inactivate. Voltage-gated Ca^{2+} channels also open upon depolarization, stimulating neurotransmitter release as well as longer term (e.g., transcriptional) effects that depend on calcium as a second messenger.

It is clear from the roles of voltage-gated ion channels in the neuronal action potential that channels must be selective for a specific ion and must open and inactivate within the proper voltage range. The conformational changes that lead to opening and closing of channels are known as “gating” and occur in response to stimuli such as ligands or voltage. Selectivity and gating depend on structural elements of the channel proteins, and X-ray crystallographic studies in the last few years have provided the first high-resolution insights into the three-dimensional organization and functional properties of channels. However, before the channels can affect cellular physiology, they must fold properly, coassemble with homologous or auxiliary subunits, and reach the plasma membrane through the secretory pathway. While gating and selectivity have been tractable properties to study with electrophysiological techniques for decades, only recently have genetic, molecular biological, biochemical, and immunological approaches enabled channel biologists

to understand the pre- and posttranslational modification, assembly, and trafficking of ion channels.

With so many levels of regulation of ion channel activity, one can imagine that mutations of ion channels at critical amino acid residues could lead to various defects in channel folding, trafficking, or conduction. Indeed, a number of ion channel-related diseases, or channelopathies, have been described, including epilepsy, arrhythmia, myotonia, diabetes, and even cancer, in humans. In addition, animal models, particularly mouse and the fruit fly *Drosophila melanogaster*, have helped to reveal the physiological roles of both wild-type and mutant channels.

As the roles that channels play in pathophysiology become clearer, the importance of developing pharmacological tools to manipulate channel function grows. Nature has provided many highly specific channel blockers in the form of peptide toxins from venomous snakes, spiders, sea snails, scorpions, and other species. While useful for laboratory studies, these toxins have poor bioavailability, so the identification and development of organic small-molecule blockers and openers have become important goals of pharmacological research.

This review will give an overview of the current state of knowledge about the structures of ion channels; the mechanisms of selectivity and gating; the cell biology of ion channels, including coassembly of channel subunits with other proteins and trafficking of ion channels; the physiological functions of ion channels and their roles in disease; and pharmacological tools available for the manipulation of ion channels in vitro and in vivo. While a vast array of ion channels exist with different selectivities and mechanisms of activation, this chapter will focus on voltage-gated K^+ , Na^+ , and Ca^{2+} channels.

14.1.1 Cloning and Evolutionary Relationships

Cloning of the first voltage-gated ion channel, from the eel *Electrophorus electricus*, showed four repeats of a six-transmembrane segment (TMS) topology, with a highly charged fourth TMS in each repeat [1]. Further cloning of other voltage-gated channels indicated that an amphipathic α helix, with an arginine residue approximately every fourth residue, is a conserved motif among voltage-gated ion channels. This charged helix appeared to be a region of the channel that might be sensitive to changes in transmembrane potential.

Identification of the genes for voltage-gated K^+ channels, beginning with the *Shaker* mutant of *Drosophila* [2, 3], revealed an evolutionary relationship among voltage-gated ion channels. Whereas the Na^+ and Ca^{2+} channels contain four pseudorepeating domains (D_{I-IV}), each containing six TMSs, the voltage-gated K^+ channels are made up of only one domain of six TMSs and assemble as tetramers (Fig. 14.1). Thus, it appears that voltage-gated sodium and Ca^{2+} channels emerged evolutionarily by successive gene duplications from a K^+ channel precursor. With the sequencing of genomes of organisms from all kingdoms of life, it is clear that voltage-gated ion channel genes are both ancient and ubiquitous. The Na^+ channels of 24-TMS topology have been found in some lower organisms, such as cnidarians and ctenophores [4], and one 24-TMS voltage-gated Ca^{2+} channel is present in the yeast *Saccharomyces cerevisiae* [5]. Among the voltage-gated cation channels, only K^+ channels are present in prokaryotes, though a Na^+ channel of 6-TM topology

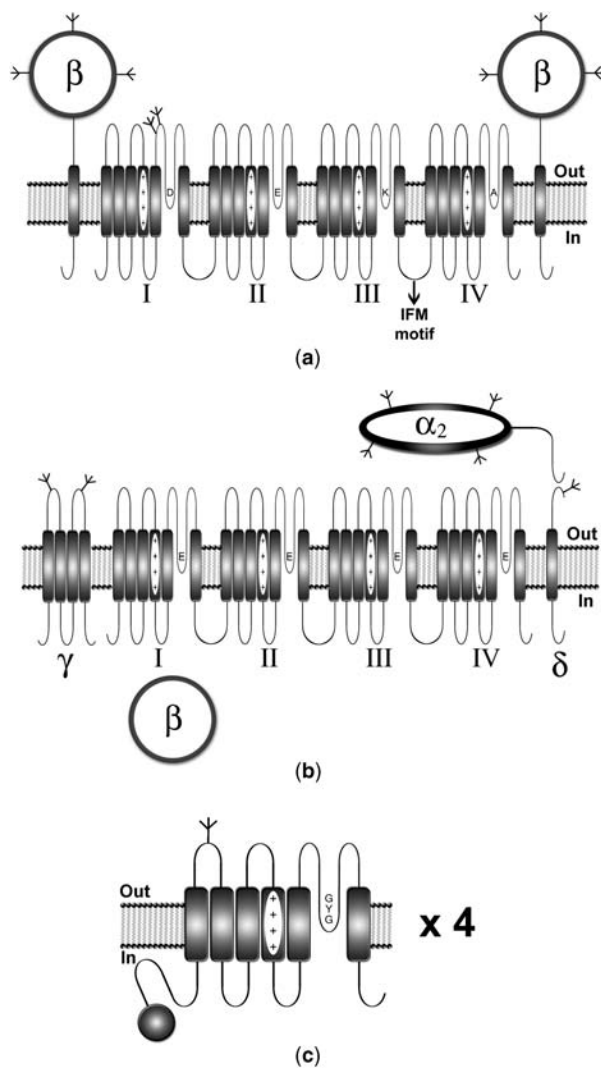


Figure 14.1 Voltage-gated ion channels. *Cylinder*: transmembrane segment. Lines indicate polypeptide chains but are not proportional to a particular polypeptide length of a channel or subunit. *Circles and ellipses*: auxiliary subunits. Pitchforks: sites of probable N-linked glycosylation. (a) Voltage-gated sodium channel and two β subunits. The DEKA selectivity filter and soleucine–phenylalanine–methionine (IFM) inactivation motif are shown. (b) Voltage-gated calcium channel along with γ , β , and $\alpha_2\delta$ subunits. The EEEE selectivity ring is shown. (c) Voltage-gated potassium channel. The GYG selectivity filter and N-terminal inactivation ball are displayed.

from *Bacillus halodurans* has been cloned and characterized [6]. Bacterial K^+ channels that can be overexpressed and purified in large quantities provided the raw materials for the X-ray crystallographic studies that show the details of ion selectivity and voltage sensing at an atomic level.

14.1.2 Nomenclature

The naming of ion channels has been an idiosyncratic process over the years, as researchers named channels for mutant or knockout phenotypes, or with acronyms derived from channel functional properties. For example, K^+ channels related to the *Shaker* channel from *Drosophila* have alternately been called Shaker, $K_v1.x$, or KCNA channels. In the interest of consistency, we will follow the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature for voltage-gated sodium (Na_v) (Fig. 14.2a) [7] and Ca^{2+} channels (Ca_v) (Fig. 14.2b) [8]. For voltage-gated K^+ channels (Fig. 14.3a), we use K_v names for the A-type and delayed rectifier channels $K_v1.x$, $K_v2.x$, $K_v3.x$, $K_v4.x$, $K_v5.1$, $K_v6.x$, $K_v8.1$, and $K_v9.x$. We use the more common literature names for the KCNQ1–5 channels and for the calcium-activated K^+ channels of large (BK), intermediate (IK), and small (SK) conductance. The more widely used *Drosophila*-derived names EAG (ether-à-go-go), ERG (ether-à-go-go-related gene), and ELK (ether-à-go-go-like gene) are employed instead of KCNH1–8 or $K_v10.x/K_v11.x/K_v12.x$ [9].

14.2 POTASSIUM CHANNELS

K^+ channels are essential in both excitable and nonexcitable cells for the control of membrane potential, regulation of cell volume, and secretion of salt, neurotransmitters, and hormones. They allow the selective, diffusional passage of potassium ions across biological membranes and are capable of up to 10,000-fold selectivity of potassium over sodium. Voltage-gated K^+ channels, unlike the related voltage-gated Na^+ and Ca^{2+} channels, are expressed not only in heart, neurons, and muscle cells but also in many nonexcitable cells.

While best known for their role in repolarizing the membrane of neurons and cardiomyocytes during an action potential, K^+ channels are, in fact, expressed in most mammalian cell types. They play a critical role in such diverse processes as epithelial salt balance across epithelial cells, particularly in the kidney and colon, T-cell signaling, and insulin release by pancreatic β cells. Accordingly, a growing number of K^+ channels are potential targets for treatment of diseases. For example, missense mutations in KCNQ2 or KCNQ3 that reduce M-channel current cause an autosomal dominant form of seizure disorder, benign neonatal familial convulsions. And a number of voltage-gated K^+ channel mutations can cause arrhythmias by delaying the repolarization phase of the cardiac action potential.

With the sequencing of the human genome, over 80 K^+ channel genes have now been identified and can be grouped into several classes based on their transmembrane (TM) topologies. The inwardly rectifying K^+ channels have two TMSs flanking the highly conserved pore region, or P loop, which confers potassium selectivity (see Section 14.2.2), and assemble as tetramers. The two-P, or KCNK channels, consist of two inward rectifier-type domains linked together and function as dimers. The voltage-gated and calcium-activated K^+ channels have an inward rectifier-type topology, containing the conserved P loop, preceded by four TM domains (five in the case of BK channels) (Fig. 14.1c). A highly charged fourth TMS functions as the voltage sensor in the voltage-gated channels. The N- and C-terminal domains of K^+

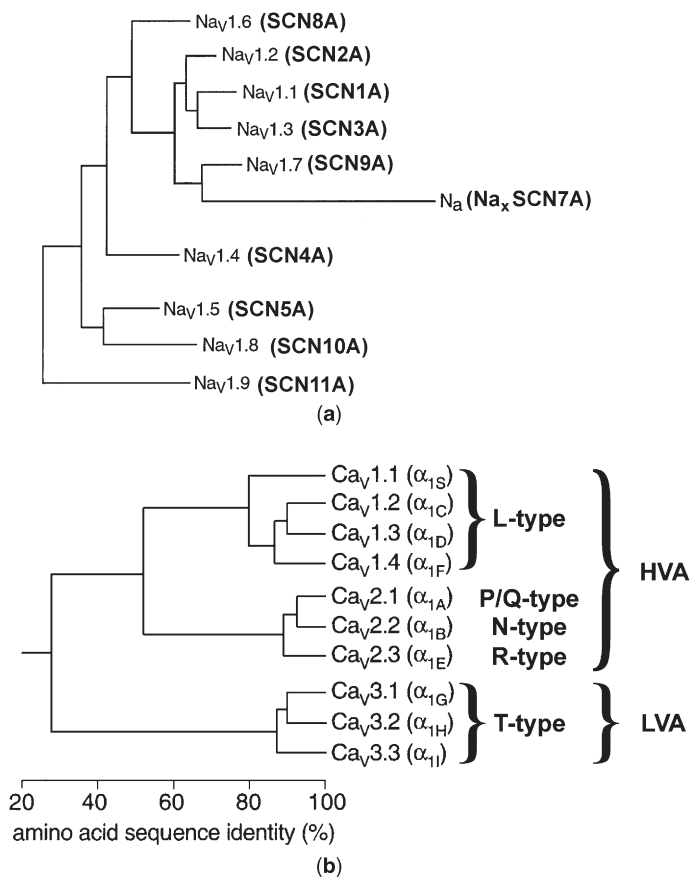


Figure 14.2 (a) Phylogenetic relationships by maximum-parsimony analysis of rat sodium channel sequences Nav1.1 through Nav1.9 and Na_x. To perform the analysis, the amino acid sequences for all of the isoforms were aligned using CLUSTAL W. The amino acid sequences in the alignments were then replaced with the published nucleotide sequences, and the nucleotide sequence alignments were subjected to analysis using the program PAUP*. Divergent portions of the terminal regions and the cytoplasmic loops between domains I–II and II–III were excluded from the PAUP* analysis. The tree was rooted by including the invertebrate sodium channel sequences during the generation of the tree, although these sequences are not shown. (Modified from [7]). (b) Sequence similarity of voltage-gated calcium channel α_1 subunits. Phylogenetic representation of the primary sequences of the calcium channels. Only the membrane-spanning segments and the pore loops (~350 amino acids) are compared. First, all sequence pairs were compared, which clearly defines three families with intrafamily sequence identities above 80% (Ca_v1, Ca_v2, Ca_v3). Then, a consensus sequence was defined for each family and these three sequences were compared with one another, with interfamily sequence identities of ~52% (Ca_v1 v. Ca_v2) and 28% (Ca_v3 v. Ca_v1 or Ca_v2). (Modified from [8].)

channels are cytoplasmic and can regulate channel electrophysiological properties and trafficking and can be a platform for phosphorylation, channel–lipid interactions, and coassembly with other proteins. In addition to these pore-forming, or α , subunits, a number of cytosolic and TM proteins coassemble with K⁺ channels and

alter channel sensitivity to various ligands or to voltage or regulate subcellular localization of the channel complex.

The last six years have brought high-resolution structures of several bacterial K^+ channels as well as cytoplasmic domains and β subunits of several mammalian channels. The structure of KcsA in 1998 [10] showed the atomic details of potassium coordination by the selectivity filter (Fig. 14.3b). While the KcsA structure shows a channel in the closed state, the structure of the calcium-activated MthK [11] revealed the conformation of an open channel. A structure of the voltage-gated K^+ channel, K_v AP, was published in 2003 [12], but there is still much debate regarding the organization of TM helices of this class of channels and the conformational changes involved in voltage gating [13]. The increasing use of structural biology as a tool for studying ion channels will allow for more detailed understanding of gating, selectivity, disease-causing mutations, and the interactions between channels and the drugs that modulate them.

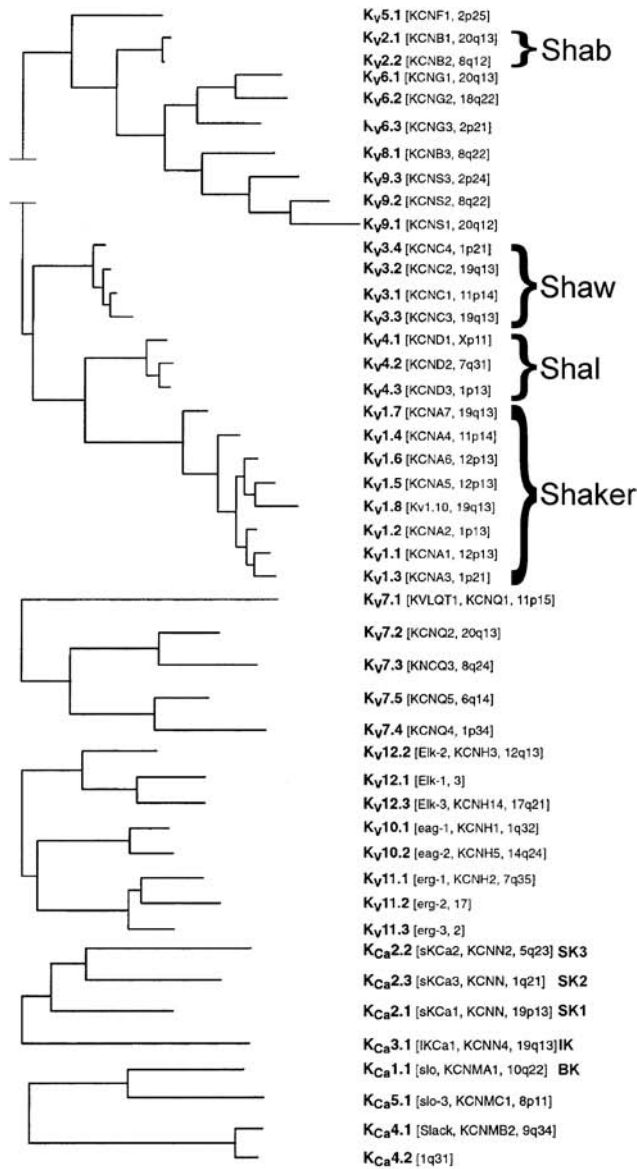
A vast pharmacology exists for K^+ channels, including many peptide toxins isolated from microbes and venomous animals and therapeutically more useful small organic compounds. Many of these agents act on multiple K^+ channels subtypes, on account of the conserved structural elements among K^+ channels, while others show potent specificity for a single channel subtype.

14.2.1 Voltage-Gated K^+ Channels

Voltage-gated K^+ channels are generally divided into subgroups depending on sequence homology and the ability to heteromultimerize. The three Shal K^+ channels (K_v 4.1–4.3, or KCND1–3), for example, can coassemble with each other but not with Shaker (K_v 1.1–1.8, or KCNA1–8) K^+ channels. The K_v channels activate upon depolarization and inactivate through either fast or slow inactivation and are also known as delayed rectifiers, since upon depolarization they repolarize the membrane after a delay. Other voltage-gated K^+ channels, the KCNQ channels and KCNH [human ether-à-go-go-related gene (HERG), EAG, ELK] channels, activate more slowly and are known as slow delayed rectifiers. While electrophysiological studies have demonstrated the diverse activation and inactivation properties of voltage-gated K^+ channels, high-resolution structural studies and spectroscopy are beginning to provide detailed molecular models for the voltage-dependent properties of these channels.

14.2.2 Selectivity

The region of K^+ channels responsible for selectivity, the P loop, was identified more than a decade ago by sequence alignment and mutagenesis studies, and a tripeptide motif Gly–Tyr–Gly was found to be crucial for distinguishing potassium from other monovalent cations [14]. It was not until MacKinnon and colleagues solved the crystal structure of the KcsA channel from *Streptomyces lividans* [10] that the geometry of potassium coordination by the channel pore became clear (Fig. 14.3b). The four subunits of the K^+ channel symmetrically surround the permeating potassium ions of the structure, with the channel reaching its narrowest point at the GYG region of the P loop. In this region, known as the selectivity filter, rings of four carbonyl oxygens coordinate potassium ions as they move through the



(a)



constriction in single file. The oxygens, in effect, mimic the hydration sphere of a potassium ion in solution and therefore lower the dehydration energy required to move the ion from bulk solvent into the protein channel. The geometry of the pore provides a more favorable coordination sphere for potassium than for sodium, which has a smaller atomic radius and a higher dehydration energy. Some have argued recently, though, that the channel pore may not be rigid and that the nature of carbonyl dipole moments themselves favor potassium over sodium [15].

Conduction occurs in the direction of the electrochemical gradient for potassium at rates approaching the diffusion limit. Repulsion between closely spaced potassium ions in the selectivity filter facilitates such rapid conduction [10]. The narrow-selectivity filter makes up less than a third of the distance of the conduction pathway, and the rest of the channel pore is wide enough to contain water molecules that hydrate ions as they pass through the inner cavity. Thus, each ion is only briefly dehydrated. Ions in the water-filled cavity below the selectivity filter are further stabilized by the orientation of negative dipoles of the four pore helices toward the cavity.

14.2.3 Voltage Activation

With the cloning and sequencing of the first voltage-gated K^+ channels, *Shaker* from *D. melanogaster* in 1987 [3], and the previously cloned voltage-gated sodium and Ca^{2+} channels, it became clear that the charged S4 TMS was likely to be important for voltage sensing. Electrophysiological studies had already shown that a “gating current” of 12–13 charges precedes the conduction of ions through the channel, indicating that a charged part of the channel protein moves across the TM electric field in a voltage-dependent manner [16]. Site-directed mutagenesis of basic residues in S4 confirmed the importance of S4 in voltage gating [17]. Later experiments showed that acidic residues in the S2 and S3 segments are important for stabilizing S4 basic residues within the membrane [18]. Based on the presumed topology of the channel and the magnitude of gating currents, several models for voltage sensor movement were proposed [4]. The S4 helix might undergo a simple translation, altering the register of S2–S4 and S3–S4 interactions and exposing some S4 residues to the extracellular solution. Alternatively, voltage sensing could be a combination of helical translation and rotation of S4. Resonance energy transfer experiments measured relatively small changes in the distance of S4 segments relative to one another, results not consistent with dramatic conformational changes [19, 20]. A way to reconcile the measured distances with the necessity of moving 12–13 charges through the TM electric field is a thinning of the membrane around S4 such that four basic residues could move from the cytoplasmic side of the membrane to the extracellular side with only a modest translation and rotation.

Figure 14.3 (a) Phylogenetic trees of the K_V and K_{Ca} channel families. An amino acid sequence alignment made using CLUSTAL W was subjected to analysis by maximum parsimony (PAUP*). Only the hydrophobic core region of the alignment was used for analysis. (Modified from [9].) (b) KcsA structure. Two subunits of KcsA are shown. The GYG motif required for K^+ selectivity is highlighted. Figure originally prepared with MOLSCRIPT and RASTER-3D. (Modified from [10].)

The structure of the bacterial voltage-gated K^+ channels K_vAP showed an unexpected orientation of S1–S4, with an S3–S4 voltage-sensing “paddle” on the outer edge of each subunit and touching lipid [12]. The data led MacKinnon and colleagues to propose that the voltage paddle is flexible and can swing across the bilayer upon voltage activation [21]. A caveat of the crystallization procedure, however, was the use of an antibody against the S3–S4 region to cocrystallize with the channel. The antibody likely distorted the voltage-sensing portion of the channel so that it may not be resting in its normal position for the depolarized state (assuming that crystallization in detergent mimics the 0 mV state of the channel). In addition, parts of the structure that appear to be on the cytoplasmic side of the membrane in the structure are glycosylated in homologous mammalian channels, suggesting that they are unlikely to move through the membrane [13]. Thus, the mechanics of voltage sensing are still an open question that will require more high-resolution structural and computational studies before it can be answered definitively.

14.2.4 Gating

Diverse stimuli can cause channel opening and closing, a process known as “gating,” and their effects must ultimately affect potassium movement through the permeation pathway of the channel. Structural and spectroscopic studies coupled with measurements of the solvent accessibility of residues in open versus closed states have identified two “gates” that restrict ion permeation. One is at the cytoplasmic face of the channel, where the inner pore-lining helices constrict and can block ion passage through the narrow opening. This region was mapped through the differential accessibility of cysteine-modifying reagents to cysteine mutations in S6 in the open and closed states [22]. Structures of the KcsA channel, which is presumably in the closed state [10], and MthK, a calcium-sensitive channel in the open state [11], confirmed this result. The KcsA structure has straight pore-lining helices that constrict at the cytoplasmic side to limit access to the selectivity filter. MthK, when opened by calcium, swings open its pore helices around a glycine hinge in M2 (S6) such that there is an opening of 12 Å from the cytoplasm to the inner pore. While voltage-gated K^+ channels may make use of this glycine hinge, they also have a Pro–X–Pro motif (though K_vAP lacks this element) at the cytoplasmic mouth of the channel that acts as a hinge and may make the opening narrower than that of MthK [23]. Interestingly, this more constricted opening may explain in part the 10-fold lower conductance of K_v channels compared to MthK.

The second gate is at the selectivity filter, which can adopt a conformation that prevents permeation of ions. This kind of gating underlies C-type (slow) inactivation of K_v channels, an inactivation mechanism that persists in the absence of fast inactivation (see below) and is dependent on the extracellular potassium concentration. The structure of KcsA in the presence of low potassium (3 mM) shows a collapsed structure that coordinates fewer potassium ions than the high-potassium (200 mM) structure [24]. The structure of a bacterial inwardly rectifying K^+ channels has a slightly different pore structure and also appears to be a nonconducting form [25] that may represent a C-type inactivated conformation.

A related question for voltage-gated channels is how voltage sensing is coupled to the channel gates to allow for opening following the movement of a gating current. During voltage sensing, the S4 domain's charged residues move outward, likely

exerting tension on the S4–S5 linker. Some studies have suggested that this linker interacts with the C-terminal end of S6 in the HERG K^+ channels [26].

14.2.5 Fast Inactivation

A-type, or rapidly inactivating, K^+ channels inactivate on a scale of tens to hundreds of milliseconds after depolarization. This inhibition is mediated by a peptide of about 30 amino acids either at the N-terminus of the channel or at the N-terminus of a β subunit (Fig. 14.4a). The peptide binds to the cytoplasmic side of the K^+ channel pore and prevents ion conduction until it is reversed by repolarization [27]. Although no structure of the inactivation peptide bound to the pore has been solved, the prevailing model has been the “ball and chain.” The N-terminal ball of basic and hydrophobic residues is tethered by a chain of linker residues to the T_1 domain or β subunit and probably snakes through the space between the T_1 domain and the membrane in order to reach the pore [28]. The ball residues are not well conserved among channels, but hydrophobic residues seem to be of particular importance in binding to the pore [29].

14.2.6 Interacting Proteins

There are many cytoplasmic proteins that have been found to interact with voltage-gated K^+ channels and to alter the electrophysiological properties or localization of the pore-forming α subunits. The β subunits exist in three forms, β_{1-3} , β_1 and β_3 having several splice variants, and coassemble with $K_v \alpha$ subunits in the endoplasmic reticulum (ER) [30]. While β_1 and β_3 are capable of converting slowly inactivating delayed rectifiers to transient A-type currents (Fig. 14.4a) [31], β_2 seems primarily to increase K_v surface expression. These subunits are members of the aldo-keto

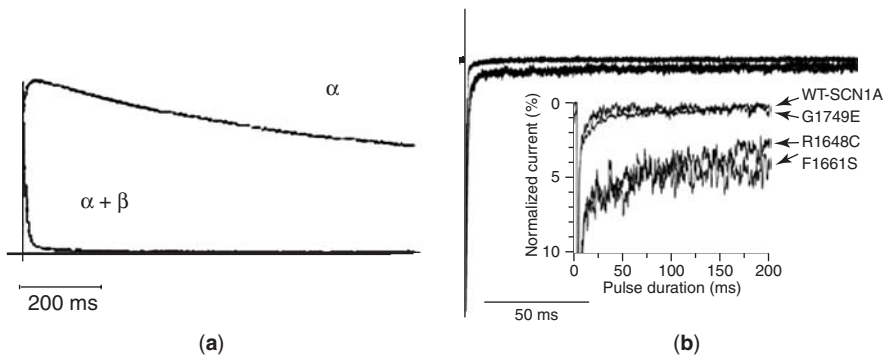


Figure 14.4 (a) Voltage-dependent activation and inactivation properties of A-type potassium currents elicited in *Xenopus* oocytes upon expression of $K_v1.1$ (α) or coexpression of α and $K_v \beta_1$ (β) subunits. Outward currents were recorded in the two-electrode voltage-clamp configuration. Current traces recorded after depolarizing pulses to +60 mV from a holding potential of −100 mV. (From [31].) (b) Noninactivating sodium currents. Sodium current was elicited by a 200-ms depolarization from −120 to 10 mV. TTX-sensitive currents were obtained by digital subtraction of sodium currents recorded before and after TTX addition. Peak sodium currents were normalized. Inset shows an expanded y axis scaled to emphasize the relative proportion of noninactivating current. (From [190].)

reductase family and bind NADPH. The function of the cofactor is not clear, and while mutation of catalytic residues seems to have no effect on trafficking of β_2 subunits, disruption of the cofactor binding site leads to improper targeting of α subunits [32]. Large-conductance calcium-activated K^+ (BK) channels interact with another type of β subunit, which has two TMSs and modifies the voltage activation and calcium sensitivity of the BK α subunits.

A second class of proteins that directly affect voltage-gated potassium activity is the KChIPs (K^+ channel interacting proteins), which interact primarily with members of the K_v4 (*Shal*) subfamily. KChIPs are cytoplasmic calcium binding proteins that have four EF-hand Ca^{2+} binding motifs and bind to the N-terminal domain of $K_v4.x$ channels. KChIPs1–3 affect α -subunit activity similarly, by increasing current density, shifting the voltage activation curve to more hyperpolarized potentials, speeding up recovery from inactivation, and slowing the time constant of inactivation [33]. KChIP4 may compete competitively with other KChIPs, fails to increase current density, and can remove rapid inactivation from $K_v4.x$ currents [34].

KCNE (MinK and MiRP) proteins are a class of TM subunits that modulate voltage-gated K^+ channel function. They contain a single TM helix with a glycosylated, extracellular N-terminus and a cytoplasmic C-terminal domain. KCNE1 assembles with KCNQ1 to form the slow component of the cardiac delayed rectifier K^+ current (I_{K_s}), part of the repolarization phase of the cardiac action potential. Its effect on KCNQ1 is to slow voltage activation and increase conductance, and mutations in KCNE1 that reduce KCNQ1/KCNE1 conductance can be arrhythmogenic [35]. KCNE1 can also interact with $K_v4.3$ and decreases its activation and inactivation rates while increasing current density [36], while KCNE3 has been found to co-immunoprecipitate with and affect the electrophysiological properties of $K_v2.1$ and $K_v3.1$ [37].

Cytoskeletal and scaffolding proteins may help to target or maintain voltage-gated K^+ channels at specific subcellular locations. Filamin, an actin binding protein, colocalizes with $K_v4.2$ and may aid in the synaptic targeting of this channel [38]. Integrins, a type of cell adhesion molecule, may help to maintain $K_v4.x$ channel complexes at the neuromuscular junction [39] and interact with $K_v1.3$ in T lymphocytes. The scaffolding protein PSD-95 can mediate synaptic clustering of K_v channels with other channels and signaling proteins [40].

14.2.7 Physiological Functions, Disease Relevance, and Pharmacology

14.2.7.1 K_v Channels. The thresholds of voltage activation and localization play key roles in the physiological function of the various K_v subunits. K^+ channels that activate at subthreshold potentials will tend to resist excitation near the resting potential, while channels that activate at more depolarized potentials will tend to play a more important role in the repolarization phase of an action potential. Axonal or axon terminal K^+ channels shape action potentials and modulate neurotransmitter release, whereas somatodendritic channels affect synaptic integration and shape backpropagating action potentials.

K_v1 channels are predominantly localized to axonal nodes of Ranvier (the juxtaparanodal region) and nerve terminals [41], though they have also been found in somatodendritic membranes [42]. Their main functions at axonal locations are

repolarization during an action potential and spike broadening at the terminal in order to regulate calcium influx and thereby neurotransmitter release. K_v1 channels form sustained currents, except when they contain a $K_v1.4$ subunit or β_1 subunits that confer fast inactivation. $K_v1.5$ is expressed in the heart, where it makes up the ultra-rapidly activating K^+ current [43], a part of the repolarization phase of the cardiac action potential, while $K_v1.3$ modulates T-lymphocyte activation by promoting calcium influx at hyperpolarized potentials [44].

K_v2 channels produce sustained currents that are localized predominantly in the cell body and dendritic membranes. $K_v2.1$ has a more restricted distribution in the soma and proximal dendrites [45], while $K_v2.2$ is found along the length of dendrites [46]. The α subunits $K_v5.1$, 6.1–6.3, 8.1, and 9.1–9.3 are all nonfunctional when expressed alone in heterologous systems. However, many of them can coassemble with K_v2 channels, acting as dominant negatives in some cases and increasing conductance in others [47, 48].

The four K_v3 channels can make up either transient or sustained currents and have more depolarized thresholds for voltage activation (>-10 mV) than most other K_v channels. Coupled with their rapid deactivation (within milliseconds), this high threshold makes these channels suited to their role in high-frequency spiking neurons [49]. Accordingly, K_v3 expression has been observed in most types of high-frequency firing neurons, such as neocortical interneurons [50] and auditory principal neurons [51].

The three K_v4 channels all form A-type currents that are expressed predominantly in somatodendritic membrane. This localization implies that K_v4 channels play a role in synaptic integration and backpropagating action potentials, which are of particular importance for informing dendritic membranes of neuronal firing activity. Their coincidence with subthreshold postsynaptic inputs can lead to synaptic plasticity through long-term potentiation. Immunohistochemistry [52] and electrophysiological studies [53] suggest that K_v4 channels, especially $K_v4.2$, are likely to mediate the A-type currents in dendrites of hippocampal pyramidal neurons. These currents increase in density with distance from the soma, leading to a dampening of backpropagating action potential amplitude in distal dendrites [54]. The inhibition of A-type potassium currents by neurotransmitter receptor-coupled phosphorylation [55] provides a mechanism for increasing backpropagating action potential frequency and *N*-methyl-D-aspartate (NMDA) receptor activation. K_v4 channels are also responsible for the transient outward potassium current ($I_{K,to}$) during the repolarization of the cardiac action potential, as $K_v4.2$ knockout mice lack this $I_{K,to}$ [56]. This current likely consists of $K_v4.2/3$ subunits and KChIP2, since a gradient in KChIP2 expression across the ventricular wall reflects the increase in $I_{K,to}$ in the epicardium compared to the endocardium [57].

Despite their ubiquitous expression in both excitable and nonexcitable cells, K_v channel mutations have been implicated in a relatively small number of diseases. Mutations in $K_v1.1$ can cause episodic ataxia type 1 (EA-1), an autosomal dominant disorder characterized by stress- or alcohol-induced attacks of imbalance and loss of coordination. EA-1 and its associated loss-of-function mutations cause an increase in action potential duration, repetitive firing, and elevated neurotransmitter release. These EA-1 mutations yield either nonfunctional channels or channels with impaired $K_v \beta_1$ -mediated N-type inactivation [58]. An intriguing pharmacological mystery with episodic ataxia is the ability of the carbonic anhydrase inhibitor acetazolamide to relieve attacks [59]. In addition to this $K_v1.1$ disorder, $K_v4.3$, in accordance with

its importance in the cardiac transient outwardly rectifying K^+ channels, has been found to be downregulated in paroxysmal atrial fibrillation [60].

The most potent blockers of K_v channels are toxins from a variety of venomous creatures, which can block either a single channel type or a range of voltage-gated channels with high affinity. Since peptide toxins make poor pharmaceutical agents, however, the channel blockers used clinically are smaller, less specific organic compounds. The least specific blockers, Ba^{2+} , Cs^+ , and tetraethylammonium (TEA), are simply cationic species that block the conduction pathway, whether they are applied to the inner or outer face of the channel. Another general blocker of K^+ channels is 4-aminopyridine, which blocks voltage-gated channels at micromolar to millimolar concentrations and likely acts at the cytoplasmic opening of the channel [61]. A slightly more potent inhibitor is quinidine, an antiarrhythmic derivative of the antimalarial drug quinine and a voltage-dependent open channel blocker [62].

Dendrotoxins are potent peptide toxins from the venom of mamba snakes that have nanomolar affinities for K_v channels. Dendrotoxin K is specific for $K_v1.1$, while α -dendrotoxin targets A-type potassium currents and β - and γ -dendrotoxins block primarily noninactivating potassium currents. The scorpion peptide margatoxin and stichodactyla toxin from sea anemone inhibit $K_v1.3$ at nanomolar concentrations and can block experimental autoimmune encephalomyelitis [63]. The prospect of $K_v1.3$ blockers as immunosuppressants has made the development of small-molecule inhibitors a promising endeavor [64, 65]. Other selective peptide toxins include BDS-I and BDS-II, from sea anemone, which block $K_v3.4$, and the spider toxin heteropodatoxin-2 and phrixotoxin-2 from tarantula, which are selective for K_v4 channels.

14.2.7.2 Large-Conductance Calcium-Activated K^+ Channels. Large-conductance calcium-activated K^+ (BK) channels are voltage dependent, have an intrinsic calcium-sensing ability and a conductance ranging from 100 to 250 pS, and play diverse roles in neuronal and nonneuronal tissues. In hippocampal neurons, they mediate both the repolarizing phase of an action potential and the fast after-hyperpolarization and respond directly to calcium influx through N-type Ca^{2+} channels [66]. And in adrenal chromaffin cells, the hypothalamic–pituitary–adrenocortical stress axis modulates epinephrine release by alternative splicing of messenger ribonucleic acid (mRNA) of BK channels, which regulate hormone release [67]. In the cochlea, a gradient of BK splice variants– β subunit complexes with varying kinetics underlies frequency tuning along the cochlear hair cell membrane [68]. The gradient makes those hair cells with more slowly gating BK channels (more β subunit) tuned to lower frequencies, while hair cells sensitive to higher frequencies have faster gating BK channels (less β subunit). Deletion of BK causes hearing loss in knockout mice and seems to be correlated with down regulated KCNQ4 expression in outer hair cell membrane that leads to degeneration of those cells [69]. BK is the most abundant channel in vascular smooth muscle, and channel composition is important in regulating vascular tone, since impaired β -subunit expression leads to increased vasoconstriction and hypertension [70]. More recently, BK channels have been found to be essential for killing of certain microorganisms by neutrophils and may function by promoting the release of microbicidal serine proteases into the phagosome [71].

There are several peptide toxins that block BK channels specifically, including iberiotoxin, slotoxin, and BmBKTx1, all of which are from scorpion venom. Charybdotoxin, another scorpion toxin, has also been used as a BK blocker, but it inhibits some voltage-gated K^+ channels as well as the intermediate conductance calcium-activated K^+ channels (IK). Nonpeptide inhibitors include the indole diterpenes paxilline, penitrem A, and verruculogen. There are several activators of the benzimidazolone family, such as NS-1619 and NS-004, that have only moderate specificity for BK. The opener BMS-204352 may limit ischemic damage during stroke [72], while NS-8 may be useful as a treatment for incontinence [73]. BK activators may also be useful in the treatment of erectile dysfunction [74], since arousal stimulates increased BK current, causing hyperpolarization and relaxation of the cavernosal smooth muscle cell membranes.

14.2.7.3 Intermediate-Conductance Calcium-Activated K^+ Channels. Intermediate-conductance (IK) and small-conductance (SK) calcium-activated K^+ channels are both voltage-insensitive six-TM K^+ channels and derive their calcium sensitivity from calmodulin, which is constitutively bound to the channel C-terminal domain. IK channels have conductances of 20–80 pS, are found mostly in nonneuronal tissues, and are often important for secretion and volume regulation. In red blood cells, increased IK channel activity can lead to cell shrinkage that promotes the concentration and aggregation of hemoglobin S in sickle cell anemia patients [75]. In T lymphocytes, IK channels are upregulated upon mitogen stimulation [76] and maintain cytoplasmic calcium concentrations necessary for activation [77]. The importance of IK in T-cell activation could make it a potentially useful target for autoimmune diseases such as multiple sclerosis and rheumatoid arthritis [78].

The only peptide toxin that inhibits IK channels potently is charybdotoxin, but, as was mentioned above, it is not specific. The antifungal clotrimazole blocks IK at micromolar concentrations but has the undesirable property of also being a cytochrome P450 inhibitor. An analog that does not affect cytochrome activity, TRAM-34, has been reported [79] and may be a more promising pharmaceutical candidate. IK blockers have been proposed as possible treatments for sickle cell anemia [80], diarrhea, and rheumatoid arthritis [81]. As is the case with $K_v1.3$ blockers, IK inhibitors may be useful as immunosuppressants. Activators include 1-ethyl-2-benzimidazolinone (1-EBIO), a more potent analog NS309, chlorzoxanone, and zoxazolamine and may be useful in treating cystic fibrosis and vascular disorders [81].

14.2.7.4 Small-Conductance Calcium-Activated K^+ Channels. SK channels have conductances of 4–20 pS and are best known for mediating the neuronal after-hyperpolarization (AHP) of medium duration. This current activates within milliseconds and has a duration on the order of hundreds of milliseconds. Depending on channel localization, the SK AHP can regulate bursting behavior in neuronal firing, set a tonic firing frequency or instantaneous firing rate, modulate rhythmic oscillations in activity, or affect spike frequency adaptation [82]. The three SK genes, *SK1–3*, have varying sensitivities to the bee venom peptide toxin apamin, a property that has helped in distinguishing these channels electrophysiologically. Apamin-blocked AHP has been proposed to play a role in learning and memory [83], and hippocampi of Alzheimer's disease patients have been found to have reduced numbers of apamin binding sites [84]. SK activity can also regulate catecholamine release in adrenal

chromaffin cells [85]. And genetic studies have suggested SK3 involvement in schizophrenia [86] and anorexia nervosa [87].

Apamin is the most potent blocker of SK channels, and more recently, the scorpion peptide toxins scyllatoxin [88] and BmSKTx [89] have been identified as selective blockers of SK channels. Tubocurarine, a plant-derived, nonpeptide inhibitor of the nicotinic acetylcholine receptor, also blocks SK channels at the same site as apamin [90]. Dequalinium and bicuculline are other nonpeptide blockers of SK channels, but the former is a mitochondrial poison and the latter blocks γ -aminobutyric acid A (GABA_A) chloride channels. Synthesis of bisquinolinium cyclophane compounds related to dequalinium has yielded blockers such as UCL-1684, which is at least 100-fold more potent than dequalinium [91]. SK activators such as 1-EBIO, NS309, and chlorzoxanone also stimulate IK channel activity and are thought to act by increasing the interaction between SK and calmodulin [92].

14.2.7.5 KCNQ Channels. Channels of the KCNQ family are voltage gated, activate slowly, and play critical roles in cardiac, neuronal, and auditory function. Mutations in four of the five KCNQ channels (KCNQ1–4) underlie genetic disorders in humans. The first member of the family, KCNQ1, was cloned as a gene associated with long-QT syndrome type 1 [93]. KCNQ1, coassembled with KCNE1, was subsequently identified as the slow delayed-rectifier current of the cardiac action potential. KCNQ1 was later also found to form a prominent current in colonic crypt cells (with KCNE3) [94] that mediates intestinal chloride homeostasis and to modulate potassium recycling in the inner ear (with KCNE1) [95].

Long-QT syndrome type 1 is caused by mutations in either KCNQ1 or KCNE1 that lead to nonfunctional or smaller currents than wild type. These mutations are located throughout the channel [96]. The reduced channel activity leads to a prolongation of the repolarization phase of the cardiac action potential and lengthening of the QT phase of the electrocardiogram. The result can be torsade de pointes, a life-threatening arrhythmia. Autosomal-dominant LQT1, or Romano–Ward syndrome, is the result of dominant negative mutations in KCNQ1 or KCNE1, while the rarer Jervall–Lange–Nielsen syndrome is recessive and causes both LQT1 and congenital bilateral deafness. Deafness results from a failure of mutant channels to maintain high endolymph K^+ concentration. Auditory stimulation leads to potassium influx into hair cells from the endolymph, followed by K^+ exit from the basal side of hair cells and recycling to the endolymph through the stria vascularis. This last step in recycling occurs through KCNQ1/KCNE1 channels, so reduced channel activity leads to reduced endolymph potassium levels and reduced endolymph potential, which diminishes sensitivity to auditory stimuli. Potassium leaves hair cells through KCNQ4 channels on the basal side of the outer hair cell membrane [97], and a loss of this channel's function can lead to outer hair cell degeneration [98]. KCNQ4 is expressed almost exclusively in the inner ear, and mutations in this channel cause nonsyndromic autosomal dominant deafness-2 (DFNA-2) [99]. Several DFNA-2-associated mutations in KCNQ4 cluster around the pore region [100].

Two other members of the KCNQ family, KCNQ2 and KCNQ3, make up the M current, a neuronal current that is activated subthreshold and inhibited by muscarinic acetylcholine receptor activation and affects neuronal excitability. While KCNQ2 and KCNQ3 expressed alone produce relatively small currents, together they form

channels with over 10 times the current expected from summing their individual conductances [101]. In addition to M_1 muscarinic receptors, other receptors that can mediate M-current inhibition include B_2 bradykinin receptors, substance P, and luteinizing hormone–releasing receptors [102]. This coupling is a way for neurotransmitters to excite the postsynaptic membrane by inhibiting a potassium conductance that resists excitation. While knockout of KCNQ2 is lethal, a mouse with a dominant-negative KCNQ2 restricted to neurons has increased hippocampal pyramidal neuron excitability and reduced spike frequency adaptation, spontaneous seizures, and behavioral hyperexcitability [103].

Mutations in KCNQ2 and KCNQ3 are associated with a seizure disorder in neonates, benign neonatal familial convulsion (BNFC), and predispose affected individuals to epilepsy later in life. BNFCs caused by KCNQ2/3 mutations can be the result of relatively modest (25%) reductions in current [104]. Mutations that cause loss of channel function in BNFCs include pore mutations and frame-shift truncations of KCNQ2 and KCNQ3 that yield nonfunctional channels and do not act as dominant negatives [96]. KCNQ4 and KCNQ5 can also coassemble with KCNQ3 and may contribute to the diversity of M currents found in the nervous system [105].

The cardiac I_{Ks} and the neuronal M current are the two major pharmaceutical targets in the KCNQ family, and there are specific pharmacologies for each. Several antiarrhythmic drugs can block KCNQ1/KCNE1 complexes selectively, particularly the chromanols HMR1556 and chromanol 293B, and can prolong the cardiac action potential and block ventricular tachycardia by prolonging I_{Ks} [106]. These blockers both are enantioselective, (–)3*R*,4*S* being the potent enantiomer in each case [107], and more potently inhibit KCNQ1/KCNE1 complexes than KCNQ1 tetramers [108, 109]. Benzodiazapines such as L-768673 and L-7 also block I_{Ks} [110]. Mefenemic acid and the chloride channel blocker DIDS nonspecifically activate KCNQ1.

Linopirdine is a relatively specific M-current inhibitor and was shown to enhance learning and cognition in a mouse model [111] but failed to show a clear affect in a human Alzheimer's disease trial [112]. The M current is also blocked by XE991, though this drug inhibits KCNQ1 and KCNQ4 as well. An activator of KCNQ2/3, retigabine, is in clinical trials as an anticonvulsant to treat epilepsy and may also be useful in treating neuropathic pain [113]. The BK activator BMS-204352 also stimulates M current.

14.2.7.6 KCNH K^+ Channels. Channels of the KCNH family include EAG, HERG, and ELK. These names derive from the channels' homology to the *Drosophila ether-à-go-go* channel and are characterized by their slow activation and direct activation by cyclic nucleotides. Another important property of these channels is that they are voltage dependent and activate at subthreshold or near-threshold potentials, suggesting that they could play a role in resting membrane potential regulation [114] and spike frequency adaptation [115]. HERG is best known for its role in the fast delayed rectifier current (I_{Kr}) of the cardiac action potential, where it carries a large portion of the outward current during repolarization. The channel has an unusual, bell-shaped current–voltage curve, with increasing currents up to +10 mV, then progressively smaller currents at more depolarized potentials [116]. This behavior is the result of slow voltage activation coupled with unusually fast C-type inactivation at depolarized potentials [117]. Mutations in

HERG are associated with long-QT syndrome, type 2, and account for the reduced potassium currents that lead to prolongation of the cardiac QT phase.

In general, the function of KCNH channels in the mammalian nervous system is not well characterized, despite the well-studied role of these channels in *Drosophila* neuromuscular function. ERG mRNA is present throughout the brain [118] and has been suggested to play a role in firing frequency and spike frequency adaptation in cerebellar Purkinje neurons [119]. The three ELK channels have predominantly neuronal expression and can coassemble with one another. Although their functional importance is unknown, their activation at potentials near the resting potential suggests a role in neuronal excitability. EAG channels are expressed predominantly in nonneuronal tissues and are notably upregulated in various tumors. Transfection of normal cells with the gene encoding EAG can lead to transformation to tumor cells [120], and EAG expression has been reported to be a marker for cervical cancer [121]. HERG also has oncogenic potential in neoplastic hematopoietic cells [122]. This unexpected role for ion channels in cell proliferation is likely related to cell cycle-dependent changes in channel activity [123].

The rapid delayed rectifier potassium current of the heart, I_{Kr} , is a major drug target for the treatment of arrhythmia, and the class III methanesulfonamide antiarrhythmics block this current carried by HERG/KCNE2. The methanesulfonamides include dofetilide, E-4031, ibutilide, D-sotalol, and MK-499. They are open channel blockers that enter the cytoplasmic mouth of the channel and bind to residues in the central cavity [124]. Class III antiarrhythmics prolong the ventricular action potential by increasing the QT phase. While these drugs can work by pore block, there are also reports that they can rescue channel mutants with folding defects and increase trafficking to the plasma membrane [125]. Unfortunately, many of these drugs are associated with drug-induced long-QT syndrome, an increased risk for torsade de pointes, and sudden cardiac death [126]. Still, the class III antiarrhythmics are useful as a therapy for atrial fibrillation or flutter [127].

Antihistamines (astemizole, terfenadine), antipsychotics (chlorpromazine, haloperidol), some antibiotics, and gastrointestinal prokinetic agents (cisapride) have also been found to cause drug-induced long-QT syndrome and ventricular arrhythmia [126]. Peptide toxins that block other voltage-gated K^+ channels are generally not effective against HERG, but recently two scorpion peptides, Ergtoxin and BeKm-1, have been identified and specifically inhibit HERG but not ELK or EAG channels [128, 129].

Although relatively little is known about EAG and ELK pharmacology relative to HERG's, EAG is a potential target for antitumor therapies, as both the antihistamine astemizole [130] and the tricyclic antidepressant imipramine [131] can inhibit EAG-mediated cell proliferation. The physiological roles of ELK channels are still unknown, and the search for their function would be aided by specific pharmacological tools.

14.2.8 Other Subfamilies of K^+ Channels: Inwardly Rectifying and Two-P K^+ Channels

The inwardly rectifying subfamily contains the basic TM unit of a K^+ channel, two TMSs flanking the P loop. The unique electrophysiological feature of these channels is inward rectification: a larger conductance at membrane potentials favoring inward current than at potentials favoring outward current. This property is the result of blockade of the cytoplasmic side of the pore by magnesium or polyamines at

depolarized potentials. These channels are open around the resting potential and resist small depolarizations but are blocked upon stronger depolarizations, such as those following the opening of voltage-gated Na^+ channels.

Inwardly rectifying K^+ channels help to set the resting membrane potential of excitable cells and play more specialized roles than other cell types. The ATP-inhibited K_{ATP} channel, for example, is an octamer of four Kir 6.1 or 6.2 subunits and four sulfonylurea receptor (SUR) subunits. Inhibition of K_{ATP} in pancreatic β cells promotes insulin release. Another class of inward rectifier is the protein-coupled inwardly rectified K^+ (GIRK) channels, which are opened by binding to the G-protein $\text{G}_{\beta\gamma}$ subunit and so are linked to the activity of G-protein-coupled receptors.

The two-P, or KCNK, channels [132] have only recently been identified and make up a subfamily of 14 members in humans. Their topology consists of two inward rectifier channels linked in tandem, so they contain two P loops per subunit and presumably assemble as dimers. Functionally, the two-P channels are thought to carry background, or leak, potassium conductances which set the resting potential of many cell types and can modulate the excitability of neurons, heart and muscle cells, and endocrine cells. While no high-affinity, specific agents for two-P channels have been identified, a number of physiological stimuli, such as mechanical stretch, acid, and polyunsaturated fatty acids, do affect channel open probability. Some neurotransmitters can inhibit two-P channels, further enhancing membrane excitability through second-messenger pathways [133]. Two-P channels may also be a target for general anesthetics, as agents such as halothane and chloroform open some KCNK channels at clinically relevant concentrations [134].

14.3 VOLTAGE-GATED SODIUM CHANNELS

Voltage-gated Na^+ channels and K^+ channels are responsible for action potential generation. In the initial phase of a neuronal action potential, small inward currents through postsynaptic ligand-gated cation channels sum to produce a threshold depolarization. This depolarization activates a large inward sodium current that depolarizes the cell even further and then inactivates within several hundred milliseconds. Voltage-gated Na^+ channels carry this large inward current, spreading the depolarization from the axon initial segment, to the nodes of Ranvier along the axon and ultimately to the nerve terminal where neurotransmitter release occurs. By contrast, voltage-gated K^+ channels are responsible for the repolarization of the membrane: They open after voltage-gated Na^+ channels and carry a large, opposing outward current that returns the membrane potential toward its resting value, reverses Na^+ channel inactivation, and allows the neuron to fire another action potential.

For repetitive neuronal firing to be possible, voltage-gated Na^+ channels must be highly selective for sodium over other ions, must open rapidly in the correct range of membrane potential, must inactivate quickly, and then must recover upon membrane repolarization. In addition, the channels must be targeted to the correct subcellular locations, such as the axon initial segment and nodes of Ranvier. Voltage-gated Na^+ channels are expressed in excitable cells of the central and peripheral nervous systems, the heart, and skeletal muscle as well as several other excitable cell types. They have been found in many animal species, both vertebrates and invertebrates,

though they are absent in the nematode *Caenorhabditis elegans*. Recently, a superfamily of bacterial voltage-gated Na^+ channels has even been identified, though these Na^+ channels resemble voltage-gated K^+ channels in topology rather than their mammalian sodium-selective counterparts [6, 135].

In their groundbreaking electrophysiological studies of the squid giant axon, Hodgkin and Huxley measured sodium- and potassium-selective conductances [136] but did not yet know what cellular components mediated these ionic fluxes or if they were even separate entities. The discovery of two toxins that could inhibit the axonal sodium current but not the potassium current, tetrodotoxin (TTX) from puffer fish and saxitoxin from *Gonyaulax* marine dinoflagellates, gave the first strong evidence that sodium and potassium traversed the membrane through distinct pathways [137, 138].

A voltage-gated Na^+ channel from the electric eel *E. electricus* was the first voltage-gated ion channel to be cloned [1], and its primary structure immediately provided clues to the function of the channel. The channel is made up of four homologous repeats ($\text{D}_{\text{I-IV}}$) of six putative TMSs (Fig. 14.1a), each repeat having a cluster of positively charged residues that were proposed to be part of the voltage-sensing mechanism. The fourth TMS (S4) of each repeat has a positively charged residue approximately every fourth residue, while the determinants of ion selectivity are residues in the region between the S5 and S6 TMS (the SS1–SS2 region). The S6 transmembrane domain lines the ion conduction pathway. Inactivation of the channel appears to be mediated by an interaction between the linker between the third and fourth repeats of the Na^+ channel and the cytoplasmic ends of the S5 and S6 TMS [139, 140].

The cytoplasmic N- and C-terminal domains of the Na^+ channel, as well as cytoplasmic linker regions between the repeats, provide sites for posttranslational modifications and coassembly with cytoplasmic domains of other proteins. Mutations in many parts of the channel can interfere with the electrophysiological function or the targeting of voltage-gated Na^+ channels and lead to a number of cardiac, neurological, and skeletomuscular disorders. In the following sections, we will discuss in more detail the molecular basis for the electrophysiological and cell biological properties of voltage-gated Na^+ channels, their physiological significance in both normal and disease states, and the pharmacology of voltage-gated Na^+ channels.

14.3.1 Single Family of Voltage-Gated Na^+ Channel α Subunits

The mammalian repertoire of voltage-gated Na^+ channels consists of 10 members which are so closely related that they make up a single family (Fig. 14.2a). This is in contrast to voltage-gated K^+ channels, which can be divided into at least 12 subfamilies, and the three subfamilies of voltage-gated Ca^{2+} channels. Nine of the α subunits, $\text{Na}_v1.1$ – 1.9 , have more than 70% identity in the TM and extracellular amino acids, while the tenth channel, Na_x , is approximately 50% identical to the other nine Na^+ channels but has sequence differences in some of the key regions for selectivity, voltage sensing, and inactivation.

The brain Na^+ channel was originally isolated as a trimer of the pore-forming α subunit $\text{Na}_v1.2$ with the β_1 and β_2 auxiliary subunits [141], but in other tissues the α subunits may interact with only one or none of the cloned β subunits. There are four β subunits, of which two, β_2 and β_4 , form disulfide bonds with the α subunit; β_1 and β_3 interact noncovalently with the α subunit. These proteins have a single TMS, a

short cytoplasmic domain, and a heavily glycosylated extracellular domain. The extracellular domain has an immunoglobulin G (IgG)-type fold that is homologous to several cell adhesion proteins and is thought to mediate clustering of channels with other proteins at nodes of Ranvier and the axon initial segment as well as cell-cell contact. In addition to affecting the subcellular localization of voltage-gated Na^+ channels, β subunits can also modulate the electrophysiological properties of α subunits, such as the rate of fast inactivation and voltage dependence of activation. The effects of β subunits may help to explain functional differences between heterologously expressed α subunits and native channels.

All members of the voltage-gated Na^+ channel family share the same topology, with four pseudorepeats, each containing six putative TMSs, and cytoplasmic N- and C-termini. The fourth TMS of the first three repeats has four to five lysines or arginines, while the S4 of the fourth repeat has seven to eight positively charged residues. Current models suggest that all four S4 segments shift toward the extracellular side of the membrane during voltage activation [142]. A number of studies of the gating charge indicate that the positively charged residues may be mostly surrounded by water-filled crevices such that the entire TM electric field is compressed into a distance of a few angstroms [143]. Accordingly, relatively modest translations of the S4 segments may be sufficient to carry the entire gating charge.

14.3.2 Selectivity

While K^+ channel selectivity is determined by backbone carbonyl coordination of ions in the selectivity filter, it is charged side chains in the Na^+ channel pore that appear to be crucial for selectivity. Voltage-gated Na^+ channels are approximately 10-fold more selective for sodium over potassium and have a negligible permeability to calcium. The SS1–SS2 region of the four repeats has a conserved set of residues, D (repeat I), E (repeat II), K (repeat III), and A (repeat IV), known as the DEKA region (Fig. 14.1a). These four residues are thought to form a ring of side chains that allow for sodium permeation while excluding potassium and calcium. Mutation of the pore lysine residue of the DEKA motif eliminates the channel's selectivity among alkali metal ions and makes the channel permeable to calcium and even to large organic cations such as TEA [144]. Ca^{2+} channels have four glutamate (E) residues at the equivalent positions, and in fact, a Na^+ channel can be converted to a Ca^{2+} channel by mutating the D, K, and A of the DEKA region to glutamates [145]. Voltage-gated K^+ channels are presumed to have a fourfold rotationally symmetric pore, since they are composed of four equivalent monomers, and the coordination of potassium resembles the geometry of a hydrated potassium ion [146]. By contrast, the Na^+ channel pore clearly uses the asymmetry afforded by four nonequivalent repeats in order to discriminate among ions.

14.3.3 Inactivation

Na^+ channel inactivation allows for the recovery of the resting membrane potential and for repetitive firing, and many disease mutations of Na^+ channels affect this aspect of channel activity. Voltage-gated Na^+ channel inactivation can be divided into a fast inactivation, which occurs within milliseconds of channel opening, and slow inactivation, which occurs on a time scale of hundreds of milliseconds to

minutes. Although inactivation depends on voltage activation of the channel, inactivation itself does not require the voltage-gated migration of any charged residues [147]. Rather, it appears to be a conformational change that leads to blockage of the conduction pore by a cytoplasmic region between the third and fourth repeats of the channel.

Fast inactivation of voltage-gated Na^+ channels is essential for proper firing of action potentials and for preventing backward propagation of action potentials along the axon. It limits the duration of the depolarization mediated by sodium influx and gives a directionality to the action potential: Once the Na^+ channels at a given node of Ranvier have opened and inactivated as part of an action potential, the action potential will propagate only in the direction of open Na^+ channels, toward the axon terminal. Three residues in the III–IV linker region are critical for inactivation and have been termed the IFM (isoleucine–phenylalanine–methionine) motif (Fig. 14.1a) [139, 148]. These residues likely dock to the inner mouth of the channel, since mutations of residues in the cytoplasmic end of the DIV S6 TMS [149] and in the DIV S4–S5 loop [150] can disrupt fast inactivation.

While fast inactivation directly affects action potential duration, slow inactivation plays a role in spike frequency adaptation and may even be important for memory of previous channel activity [151]. Near the resting potential, very few channels undergo fast inactivation, but slow inactivation can be a factor in regulating the duration of openings in this range. Thus, slow inactivation can decrease membrane excitability by keeping voltage-gated Na^+ channels closed in the steady state. The P region, or SS1–SS2, appears to be important for slow inactivation, since a residue in this part of the h $\text{Na}_v1.5$ (D_{II}) was found to be a critical determinant of the probability of entry into a slow-inactivated state [152]. As is the case with voltage-gated K^+ channels, voltage-gated Na^+ channel slow inactivation may be due to a collapse of the pore in response to other conformational changes within the channel.

14.3.4 Trafficking

A well-characterized example of voltage-gated Na^+ channel trafficking is the localization of $\text{Na}_v1.2$ at the axon initial segment of neurons. Although the β subunits are involved in adhesion and channel trafficking, they do not themselves target Na^+ channels to the axon. Rather, the α subunit contains several motifs that allow for its proper localization. A dileucine motif in the C-terminal domain is responsible for internalization of the channel in somadendritic membrane but not axonal membrane [153]. Axonal channels may be trapped by the ankyrin G– β -IV spectrin complex, while the Na^+ channels in nonaxonal membrane are untethered and retrieved by endocytosis [154]. While the C-terminus is sufficient for axonal targeting, it cannot localize $\text{Na}_v1.2$ at the initial segment of the axon. The channel has an acidic cluster in the cytoplasmic II–III linker that acts as a retention motif for the axon initial segment [155]. This motif binds to the clustering protein ankyrin G and is conserved in $\text{Na}_v1.1$, 1.3, 1.4, 1.5, and 1.6.

Interactions between ankyrin G and β -spectrin help Na^+ channels to cluster at high density in the postsynaptic membrane of neuromuscular junctions [156]. Ankyrin G is also essential for targeting of voltage-gated Na^+ channels to the axon initial segment and nodes of Ranvier, as mice with ankyrin G knocked out fail to properly localize axonal Na^+ channels [157]. Neurofascin and NrCAM are cell

adhesion proteins that cluster with ankyrin G at the axon initial segment and are important for Na^+ channel accumulation at nodes of myelinating cells [158]. Cell adhesion molecules prevent channels from diffusing away from nodes of Ranvier and the axon initial segment.

14.3.5 Physiological Functions

Of the nine members of the $\text{Na}_v1.x$ family, seven are expressed in the central and/or peripheral nervous system; $\text{Na}_v1.5$ is found primarily in heart muscle and $\text{Na}_v1.4$ is localized to skeletal muscle cells. Na_x has a wider distribution and has been identified in heart, uterus, glia, the peripheral nervous system, and smooth muscle.

Within neurons, Na^+ channels are often localized at the axon initial segment and nodes of Ranvier, where they play an essential role in the propagation of action potentials. These channels generally carry transient, fast-inactivating sodium currents that allow for rapid recovery and repetitive firing of neurons. Somatic integration of excitatory synaptic inputs causes action potential initiation at the axon initial segment. The insulating properties of myelin allow the action potential to jump rapidly from node to node and ultimately to the synaptic terminal, where the opening of voltage-gated Ca^{2+} channels stimulates neurotransmitter release.

Although some voltage-gated Na^+ channels are selectively retrieved from somatodendritic membrane, Na^+ channels are still present in dendrites. At post synaptic dendritic sites, Na^+ channels may amplify excitatory postsynaptic potentials as they travel toward the soma. In addition, action potentials can backpropagate from the soma to the dendrites, and the efficiency of backpropagation is dependent on the dendritic Na^+ channel density [159]. In Purkinje neurons, for example, dendritic Na^+ channel density decreases steeply with distance from the soma [160], so backpropagating action potentials dissipate quickly. In mitral cells or hippocampal CA1 neurons [159, 161], however, there is a higher ratio of Na^+ to K^+ channels in the dendrites, allowing for regenerative action potential backpropagation. The coincidence of subthreshold postsynaptic stimulation and backpropagating action potentials is thought to underlie some forms of synaptic plasticity. The calcium influxes mediated by this coincidence could mediate long-term potentiation (LTP) and other transcriptional changes in neuronal activity.

While the best characterized voltage-gated sodium currents are transient and fast inactivating (Fig. 14.4b), sodium currents with different inactivation properties are important for modulating excitability. Persistent sodium currents (Fig. 14.4b) are present at subthreshold potentials, where channels can open but fast inactivation does not occur. Their activation can effectively depolarize the cell such that it is closer to the threshold for action potential firing. More substantial depolarization by a persistent sodium current, however, can lead to resting inactivation of Na^+ channels and decreased excitability. This distinction underlies the ability of some skeletal muscle Na^+ channel mutations that disrupt fast inactivation to cause myotonia (hyperexcitability), while others cause paralysis (hypoexcitability). Inhibiting persistent sodium currents also decreases excitability as K^+ channels that are open at rest push the membrane toward more hyperpolarized potentials. Persistent currents have been found electrophysiologically in central [162] and peripheral neurons [163] and are thought to be mediated by $\text{Na}_v1.6$. There are other reports of TTX-resistant persistent sodium currents as well [164].

In addition to the rapidly inactivating and persistent sodium currents, a resurgent sodium current has also been characterized [165]. It appears after a large depolarization, such as an action potential, followed by a return to a slightly depolarized potential (-40 mV). The resurgent current increases slowly and decays slowly and might facilitate repetitive firing. The molecular identity of this current is not known, though mice lacking $\text{Na}_v1.6$ also lack the resurgent current [166].

Several Na^+ channel family members are expressed predominantly in the peripheral nervous system, particularly in sensory neurons, and are thought to play a role in pain sensation. The Na^+ channel-blocking activity of local anesthetics provides further evidence for the role of voltage-gated sodium currents in nociception. $\text{Na}_v1.7$ is expressed at the terminal of sensory neurons, while $\text{Na}_v1.8$ is exclusively expressed in sensory, especially nociceptive, neurons. Both channels respond to inflammatory factors involved in hyperalgesia, like nerve growth factor (NGF) [167, 168], which increases expression of these channels in response to tissue damage and leads to inflammatory pain sensation. Primary erythromalgia, a chronic human inflammatory disorder, which causes intermittent extreme pain and redness in the feet and hands, maps to the $\text{Na}_v1.7$ locus [169], and the associated mutations lead to hyperexcitability of nociceptive dorsal root ganglion (DRG) neurons [170]. Accordingly, mice with $\text{Na}_v1.7$ deleted from sensory neurons have increased mechanical and thermal pain thresholds and decreased inflammatory pain responses [171]. $\text{Na}_v1.8$ is also likely to play a role in inflammatory pain on account of its upregulation by NGF and the analgesic effects of $\text{Na}_v1.8$ antisense in models of inflammatory pain [172].

$\text{Na}_v1.3$ is generally expressed in the adult central nervous system, but upon nerve damage such as axotomy this channel and the β_3 subunit can be upregulated in sensory neurons [173]. This expression of $\text{Na}_v1.3$ may be an important cause of hyperexcitability and neuropathic pain following nerve injury. Its upregulation can be suppressed by glial-derived neurotrophic factor (GDNF) [174], a factor associated with analgesia. $\text{Na}_v1.9$, by contrast, provides a persistent sodium current that is downregulated after axotomy [175]. Since $\text{Na}_v1.9$ is activated at more hyperpolarized potentials compared to other Na^+ channels and has relatively slow kinetics of activation and inactivation [164], a decrease in its expression could remove resting inactivation of other Na^+ channels and thereby increase excitability. Thus, activation of $\text{Na}_v1.9$ might be an effective way to minimize neuropathic pain.

In the central nervous system, changes in the localization of neuronal channels in myelinated neurons may play a role in the degenerative processes of multiple sclerosis (MS). $\text{Na}_v1.6$ is usually localized to the nodes of Ranvier, but its distribution throughout the axon becomes more diffuse during the demyelination characteristic of MS. $\text{Na}_v1.6$ is also upregulated and colocalizes with the sodium–calcium exchanger as well as with β -amyloid precursor protein, a marker for axonal damage, in MS-damaged neurons. The persistent current mediated by $\text{Na}_v1.6$ may cause increased calcium influx into the cell via the sodium–calcium exchanger and thus provide a mechanism for toxicity [176].

The predominant voltage-gated Na^+ channel subunit in heart cells is $\text{Na}_v1.5$, which plays a key role in the depolarization and plateau phases of the cardiac action potential. A number of arrhythmias and other cardiac diseases are due to mutations in $\text{Na}_v1.5$, and drugs from the class I antiarrhythmics are blockers of the heart voltage-gated Na^+ channel. Brain Na^+ channels $\text{Na}_v1.1$ and 1.3 have also been

identified in the sinoatrial node of the heart, where no $\text{Na}_v1.5$ is expressed. The associated currents are TTX-sensitive and their blockade results in greater heart rate variability and lower spontaneous heart rate [177].

The $\text{Na}_x \text{Na}^+$ channel has only 50% identity to members of the $\text{Na}_v1.x$ family and notably has fewer charges in the S4 segments and low conservation of residues in the III–IV linker region that mediates fast inactivation. A mouse with this gene knocked out has a reduced capacity to regulate NaCl intake under both high- and low-salt conditions [178]. Na_x is expressed, among other places, in the circum ventricular organs of the brain, which control salt and water homeostasis, consistent with its role in osmoregulation.

14.3.6 Channelopathies

Na^+ channels expressed in skeletal muscle, heart, and the nervous system have been implicated in a number of genetic disorders, and Na^+ channel activity plays a role in many other disease states. Inherited mutations that affect channel activity most frequently change the inactivation properties of the Na^+ channel, rendering the cells where they are expressed either more or less excitable than they would normally be. Other mutations affect voltage-dependent activation or assembly with other proteins required for proper channel function.

Mutations in the skeletal muscle Na^+ channel, $\text{Na}_v1.4$, are associated with several channelopathies, including paramyotonia congenita, potassium-aggravated myotonia, hyperkalemic periodic paralysis, and hypokalemic periodic paralysis. Myotonias are characterized by difficulty with muscle relaxation, triggered by exercise, cold, or a rise in plasma potassium levels, and produce repetitive activity in an electromyogram. The most common mutations are T1313M in the III–IV linker and R1448H/C in the S4 of domain IV, both of which slow fast inactivation [179]. Both types of mutations lead to a persistent current that slightly depolarizes muscle cells, enough to decrease the firing threshold but not sufficient to lead to resting inactivation of the Na^+ channel population. Potassium-aggravated myotonia is frequently found to be associated with the G1306E/V/A mutations in the III–IV linker [180]. These mutations in the fast-inactivation loop slow inactivation and lead to a persistent current with more frequent, longer openings.

Periodic paralysis, in contrast to myotonia, is a disorder caused by reduced muscle excitability and is manifested by a silent electromyogram. Hyperkalemic periodic paralysis (HyperPP) can be triggered by stress, fasting, potassium-rich foods, or exercise. The episodic muscle weakness associated with the disorder is the result of incomplete channel inactivation, which leads to a persistent sodium current that can depolarize the resting membrane potential from -90 mV to as high as -50 mV [96 p. 79]. This depolarization causes most of the Na^+ channels to be inactivated at rest and makes them unable to initiate an action potential, explaining the silent electromyogram. Approximately 90% of families with this disorder have a mutation in S6 (D_{II}), T704M, which causes a 10-mV leftward shift in the voltage-dependent opening of the channel [96]. Thus, channels open at more negative potentials, well outside their inactivation range, creating a persistent current that depolarizes the resting membrane potential. Mutations of $\text{Na}_v1.4$ can also lead to hypokalemic periodic paralysis. These mutations lie in D_{II} S4 and enhance both fast and slow inactivation [181].

The cardiac Na^+ channel, $\text{Na}_v1.5$, is associated with several inherited arrhythmic disorders, including long-QT syndrome type 3, Brugada syndrome, and conduction disorder. Long-QT syndrome is caused by a slowed repolarization of the cardiac action potential, manifested by a lengthening of the QT phase of the electrocardiogram. This arrhythmia can lead to lower ventricular filling, ventricular fibrillation, decreased cardiac output, and death. All $\text{Na}_v1.5$ mutations that cause long-QT syndrome type 3 result in defects in fast inactivation [182]. The most severe of these is the $\Delta\text{KPQ1505-7}$ deletion in the III–IV loop [183]. Impaired inactivation prolongs the plateau of the cardiac action potential with a persistent sodium current, resulting in slower repolarization and a QT interval that is 2–5% longer than normal [96]. Class Ib antiarrhythmics such as mexiletine have been used to reduce the persistent sodium current of the mutant channel.

Brugada syndrome [184] is a cardiac arrhythmia that elevates the ST segment of the electrocardiogram, substantially increasing the risk of idiopathic ventricular fibrillation in affected individuals. The elevated ST segment is the result of a voltage gradient between the epicardium and the endocardium: The loss of sodium current has a greater effect on the epicardium, on account of its counteracting the transient outward potassium current ($I_{K_{to}}$), than on the endocardium, where this potassium current is less prominent. The epicardium thus repolarizes prematurely relative to the endocardium, resulting in an arrhythmogenic transmural voltage gradient. Disease mutations are found at amino acids throughout the channel and generally render the channel nonfunctional [185].

In the central nervous system, mutants of both the Na^+ channel α subunit $\text{Na}_v1.1$ and the β_1 subunit have been found to cause generalized epilepsy with febrile seizures. The β -subunit mutation (C121W) maps to a cysteine residue in the extracellular domain that is involved in a disulfide bond which is essential for proper folding of the domain [186]. The α -subunit mutations decrease interaction with β_1 (D1866Y), causing a right shift in the voltage dependence of inactivation and a greater persistent current, or can affect the voltage-dependence of inactivation (I1656M and R1657C) by uncoupling voltage sensor movements from fast inactivation [187–189]. Another form of epilepsy, severe myoclonic epilepsy of infancy (SMEI), has also been mapped to several sites on the *scn1a* ($\text{Na}_v1.1$) gene [190]. Mutants causing this form of epilepsy are either nonfunctional or noninactivating (Fig. 14.4b). Several missense mutations of $\text{Na}_v1.2$ can also lead to GEFS [191] or benign familial neonatal–infantile seizures [192], while nonsense mutations are correlated with intractable epilepsy such as SMEI [193]. Voltage-gated Na^+ channel mutations may also predispose individuals to autism [194].

14.3.7 Pharmacology

Several classes of toxins and small organic compounds bind to distinct sites on voltage-gated Na^+ channels, affecting channel permeation or inactivation. The first identified toxins that could specifically block Na^+ channels are TTX and saxitoxin (STX), which have varying affinities for different members of the voltage-gated Na^+ channel family. TTX is concentrated from marine bacteria by the *Fugu* puffer fish, while STX is a poison from dinoflagellates, which are ingested by various shellfish. Both bind with low nanomolar affinity to the skeletal muscle and brain channels and have much lower affinity for the cardiac and peripheral Na^+ channels $\text{Na}_v1.8$ and 1.9. The TTX/STX binding site is determined by residues in the selectivity filter of the

channel, as well as the β subunit, and not surprisingly, these toxins block the channel pore. Both toxins contain a positively charged guanidinium group that interacts with glutamate and aspartate residues in the SS2 regions. The μ -conotoxins are peptide toxins from the *Conus* marine snails and have a guanidinium group from an arginine residue that mediates binding to the same part of the channel as TTX and STX.

A second class of toxins is composed of lipid-soluble compounds such as the plant-derived aconitine, batrachotoxin (BTX), and grayanotoxin (GTX) and veratridine, which is secreted by the skin of poisonous frogs from the *Phyllobates* genus. Unlike TTX, STX, and μ -conotoxins, these lipid-soluble toxins are activators of Na^+ channels that shift voltage activation to more negative potentials and inhibit fast inactivation. Ciguatoxins (CTX) and brevetoxin (PbTx), both derived from marine dinoflagellates, make up another class of lipid-soluble toxins that activate voltage gated Na^+ channels by shifting the voltage dependence of activation to more hyperpolarized potentials and by inhibiting fast inactivation. The type I sea anemone toxin, scorpion α -toxins, and Australian funnel-web spider-derived atracotoxins prevent fast inactivation of voltage-gated Na^+ channels by binding extracellular S3–S4 and S5–S6 linkers [195]. The peptide scorpion β -toxins bind to the extracellular end of S4 D_{II} and the S3–S4 D_{II} loop and likely stabilize the open conformation of the voltage-sensing S4 transmembrane domain [196].

Other activators of voltage-gated Na^+ channels include the pyrethroid insecticides, such as dichlorodiphenyltrichloroethane (DDT). Antiepileptic drugs like phenytoin, lamotrigine, and carbamazepine are blockers of voltage-gated Na^+ channels, and the Na^+ channel-blocking action of antidepressants such as imipramine, amitriptyline, and maprotiline may mediate sedative and arrhythmogenic side effects of these drugs. Antiarrhythmic drugs of class 1b, such as mexiletine, as well as local anesthetics have been useful treatments for paramyotonia congenita and potassium-aggravated myotonia and can be neuroprotective in some cases. Antiarrhythmic drugs have not been as useful as one might hope, however, on account of their side effects: Their tendency to promote arrhythmias in many patients is of particular concern.

Local anesthetics make up a class of voltage-gated Na^+ channel blockers that act on nociceptive fibers of the peripheral nervous system. These compounds are lipid soluble and include lidocaine, bupivacaine, benzocaine and many other compounds. They cross the membrane in order to reach their site of action at the cytoplasmic mouth of the channel, where they display use-dependent block: That is, successive depolarizations increase access of local anesthetics to their binding site on the channel [197]. Unlike the blockers TTX and STX, there is relatively little variability in local anesthetic affinity among the members of the voltage-gated Na^+ channel family. Affinities do not necessarily correlate with analgesic efficacy, since repetitive firing, not current amplitude, is what underlies nociceptive transmission [198].

14.4 VOLTAGE-GATED CALCIUM CHANNELS

Voltage-gated Ca^{2+} channels are essential for mediating cellular responses to electrical activity, exemplified by the fundamental requirement of these channels in neurotransmitter release and muscle contraction. Intracellular calcium ion concentration is highly regulated and normally maintained around 100 nM, as prolonged elevation of calcium levels can trigger cell death. Voltage-gated Ca^{2+} channels play a major role in

controlling intracellular calcium concentrations. The influx of calcium into a cell induced by the opening of a voltage-gated Ca^{2+} channel can cause over 1000-fold increase in localized calcium concentration, resulting in profound changes in cellular function.

Over the decades, an impressive body of work has examined the physiological impact of calcium currents and the proteins involved in shaping these currents. The initial cloning of a voltage-gated Ca^{2+} channel as well as the biochemical isolation of an α subunit with its auxiliary subunits occurred in 1987 [199, 200]. Over the years, a total of 10 pore-forming α_1 subunits from humans have been cloned along with a wide array of auxiliary subunits: four $\alpha_2\delta$, four β , and eight γ subunits.

Like voltage-gated sodium channels, the voltage-gated Ca^{2+} channel's pore-forming α -subunit gene encodes a 24-TM protein divided into four 6-TMS pseudorepeating domains. This pore-forming α_1 subunit interacts noncovalently with the auxiliary subunits $\alpha_2\delta$, β , and sometimes γ at nonoverlapping domains of the α_1 subunit to form a voltage-gated Ca^{2+} channel complex (Fig. 14.1b).

The interactions of these subunits can have a profound impact on the function, localization, and stability of the Ca^{2+} channel. Ultrastructural studies using electron microscopy and single-particle analysis of the skeletal muscle Ca^{2+} channel ($\text{Ca}_v1.1$, $\alpha_2\delta_1$, β_{1a} , γ_1) and cardiac Ca^{2+} channel ($\text{Ca}_v1.2$, $\alpha_2\delta_1$, β_2 , or β_3) at nanometer resolution have provided some insight into the physical association of the voltage-gated Ca^{2+} channel subunits, corroborating information gleaned from biochemical studies over the past few years [201]. High-resolution crystal structures of Ca^{2+} channel components are an enticing but challenging goal that has not been fully realized. Obtaining a more structurally refined image of the Ca^{2+} channel will provide greater insight into the channel's mechanics and function and be a guide for the development of drugs to alter Ca^{2+} channel activity.

Recently, the first crystal structures of a voltage-gated Ca^{2+} channel domain were published, depicting the 18-amino-acid stretch of the cytoplasmic domain I–II linker site where the β subunits interact with the α_1 subunit, known as the α_1 interaction domain (AID) bound to different β subunits at high resolution [202–204]. These structures reveal that the AID is an amphipathic helix. The highly conserved hydrophobic residues of the AID form a complement to the hydrophobic groove of the β subunit that is most intimate with the AID. Unlike the β subunit, it is not clear where $\alpha_2\delta$ or γ interacts with the $\text{Ca}_v \alpha_1$ subunit.

There is great diversity among voltage-gated Ca^{2+} channels. From the sheer number of possible subunit combinations that assemble to form a functional channel to the range of signaling pathways involved in these channels, great challenges and opportunities exist in pharmacologically controlling these channels. Buoyed by the early success of blocking voltage-gated Ca^{2+} channels to treat hypertension, continued efforts are underway to better understand specific roles of voltage-gated Ca^{2+} channels and to develop new and better pharmacological tools.

14.4.1 α Subunit

The broadest classifications among the 10 cloned α subunits are the high-voltage activated (HVA) and the low-voltage activated (LVA) classes of subunits [205]. The HVA Ca^{2+} channels require a large depolarization to open in normal physiological conditions. Further classification of HVA channels is now possible based upon their pharmacological profile: dihydropyridine (DHP)-sensitive channels (L type,

Ca_v1.1–1.4), ω -agatoxin IVA-sensitive channels (P and Q type, Ca_v2.1), ω -conotoxin GVIA-sensitive channels (N type, Ca_v2.2), and SNX482-sensitive channels (R type, Ca_v2.3) [8]. The remaining three cloned Ca²⁺ channels are the LVA-type channels (Ca_v3.1–3.3), also known as T-type channels, and have no known specific blockers at this time (Fig. 14.2b). These channels open at membrane potentials close to rest and have a small single-channel conductance [206, 207] but can collectively influence the excitability and signaling properties of a neuron.

Voltage-gated Ca²⁺ channels are able to selectively allow calcium entry through the cell membrane and exclude the passage of other ions, including sodium, which is roughly the same size as a calcium ion. A ring of four glutamates, one from the pore lining P loop of each pseudorepeat, is the crucial site for calcium selectivity and is conserved among all of the HVA α subunits [208]. The acidic side chains of this EEEE ring motif are believed to project into the pore interior of the Ca²⁺ channel, allowing the negatively charged carboxylate oxygens to coordinate a calcium ion. Even a subtle mutation of a single glutamate to aspartate or glutamine reduces the calcium affinity of the voltage-gated Ca²⁺ channel [209]. LVA Ca_v3 channels have a similar selectivity ring, but with two glutamates and two aspartates instead of four glutamates forming the ring.

There is a proposed hinged-lid model for the fast inactivation of voltage-gated Ca²⁺ channels [210]. S6 of D_{I-IV} acts as a docking site for the D_{I-II} linker region to block the conduction of calcium reminiscent of the ball-and-chain model seen in sodium and potassium voltage-gated channels.

14.4.2 β Subunits

Of the four known cytosolic β subunits, β_{1a} was the first discovered when it was copurified with the initial isolation of Ca_v1.1 in skeletal muscle [211]. Over the years, characterizations of the four β subunits have revealed numerous splice variants with distinct properties [212]. β_{1b} and β_{2a} , for example, have the ability to independently associate with the plasma membrane via acidic motifs or palmitoylation [213, 214].

Sequence analysis and the recent crystal structures of β_2 , β_3 , and β_4 show that β subunits are part of the membrane-associated guanylate kinase (MAGUK) family [215]. SH3, a protein–protein binding motif, and a guanylate kinase motif are the two main motifs of MAGUK proteins, although the enzymatic capability of the guanylate kinase has been modified in β subunits and is now involved in establishing protein–protein interfaces that modulate Ca_v α_1 Ca²⁺ channel function.

The ability of β subunits to physically interact with the AID was thought to be primarily mediated by a 41-amino-acid region of the β subunit known as the β -interacting domain [216]. However, crystal structures of the AID bound to a β subunit have revealed a more accurate portrayal of this region that binds to the AID, dubbed the AID binding pocket (ABP). The ABP is located at the ATP coordination site of the GK domain, but a conserved tryptophan in the AID allows the β subunit to bind to the α subunit instead of ATP.

The β subunits have a number of profound effects on the trafficking, kinetics, and voltage properties of the voltage-gated Ca²⁺ channel [217]. With the exception of some truncated splice variants, β subunits increase the functional expression of HVA Ca²⁺ channels. Almost all voltage-gated Ca²⁺ channel α subunits have an ER

retention signal located in the I–II cytoplasmic loop and β subunits shield this signal to promote exit from the ER and surface expression of the channel [218]. The β subunits also influence Ca^{2+} channels at the plasma membrane and are capable of increasing the mean open time of Ca^{2+} channels as well as shifting the voltage dependence of HVA Ca^{2+} channel activation, making the Ca^{2+} channel more apt to open when a cell depolarizes. The voltage dependence of steady-state inactivation is also shifted to more hyperpolarized potentials by β subunits, with the exception of N-terminal palmitoylated β_{2a} . Palmitoylated β_{2a} retards the inactivation of $\text{Ca}_v1.2$, 2.2, and 2.3.

14.4.3 $\alpha_2\delta$ Subunits

There are four known mammalian $\alpha_2\delta$ subunits that interact with voltage-gated Ca^{2+} channel α subunits. Expanding on this molecular diversity, at least five splice variants of $\alpha_2\delta_1$ ($\alpha_2\delta_{1a}$ to $\alpha_2\delta_{1e}$) and three of $\alpha_2\delta_2$ ($\alpha_2\delta_{2a}$, $\alpha_2\gamma_{2b}$, and $\alpha_2\delta_{2c}$) have been detected with a tissue-specific distribution pattern. A salient feature of the $\alpha_2\delta$ subunit is that it is proteolytically cleaved and the resulting α_2 and δ peptides are linked together via disulfide bridges. The entire α_2 subunit is located extracellularly and the δ subunit is a single-TM protein that acts as its anchor [219].

The α_2 subunit is heavily glycosylated and presumably this glycosylation ultimately influences the trafficking and stability of the voltage-gated Ca^{2+} channel. Indeed, $\alpha_2\delta$ generally increases the surface expression level of voltage-gated Ca^{2+} channels [220]. Depending on the $\text{Ca}_v \alpha_1$ and β subunits coexpressed with $\alpha_2\delta$ in a heterologous expression system, $\alpha_2\delta$ is also capable of accelerating both the activation and inactivation of calcium current. Like the β subunit, $\alpha_2\delta$ also shifts the voltage dependence of the Ca^{2+} channels to more hyperpolarized potentials. It is not clear how $\alpha_2\delta$ modulates these biophysical properties of the voltage-gated Ca^{2+} channel.

14.4.4 γ Subunits

The primary focus of research on γ subunits involved in Ca^{2+} channel function has been on its role in skeletal muscle, the only tissue known to express calcium γ subunits until recently. Since the discovery that the spontaneous mutation in the *stargazer* mouse line is a neuronal γ subunit [221], the number of γ subunits cloned has expanded to eight, and a number of these are expressed in the brain [222].

The γ subunits have four TM domains with intracellular amino and carboxyl termini. Features of some of the neuronal γ subunits are a cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP) phosphorylation consensus site and potential sites for N-linked glycosylation. The γ subunits are unique among the auxiliary subunits in not having a known influence on voltage-gated Ca^{2+} channel surface density. They also do not interfere with the $\alpha_2\delta$ or β subunits interactions with the $\text{Ca}_v \alpha$ subunit. The impact of γ subunits on voltage-gated Ca^{2+} channel electrophysiology has not been robust or clearly understood. The general consensus is that γ subunits lower the current of Ca^{2+} channels by causing a hyperpolarizing shift in the voltage dependence of inactivation.

14.4.5 Function and Pharmacology

Voltage-gated Ca^{2+} channels are expressed in all excitable cells and play numerous functional roles in a variety of signaling pathways that encode electrical activity into cellular events [4]. Although voltage-gated Ca^{2+} channels share fundamental physiological properties, the different $\text{Ca}_v \alpha$ and auxiliary subunits have specialized distributions, functions, and pharmacological sensitivities. For decades, Ca^{2+} channels have been manipulated by researchers using multivalent cations and small organic molecules. The repertoire of pharmacological manipulations has more recently been expanded using peptides mainly derived from the venoms of predatory creatures. There continue to be focused efforts on the discovery and synthesis of new compounds to influence voltage-gated Ca^{2+} channel behavior [223].

14.4.5.1 General Blockers. Certain metal ions are capable of nonselectively blocking voltage-gated Ca^{2+} channels. Cadmium ions block all HVA Ca^{2+} channels. Nickel ions were historically used to block LVA channels, but only $\text{Ca}_v3.2$ of the LVA family as well as a number of HVA channels are actually blocked by nickel [224]. Trivalent cations such as lanthanum, yttrium, and holmium are potent blockers of all voltage-gated Ca^{2+} channels. These multivalent ions block voltage-gated Ca^{2+} channels primarily by occlusion of the α_1 pore region containing the EEEE ring of glutamic acid residues. These ions also have secondary effects outside of the pore region that influence the biophysical properties of voltage-gated Ca^{2+} channel.

14.4.5.2 Ca_v1 Family. $\text{Ca}_v1.1$ expressed in skeletal muscle is directly coupled with ryanodine receptors that line the sarcoplasmic reticulum, which stores intracellular calcium. Depolarization of skeletal muscle plasma membrane opens $\text{Ca}_v1.1$ with slow kinetics, causing activation of ryanodine receptors and leading to intracellular release of calcium. The released calcium from the internal stores binds to proteins such as troponin and ultimately allows myosin to generate the force necessary for contraction. Mutations of $\text{Ca}_v1.1$ have been associated with malignant hyperthermia, a condition in which a normally healthy individual can experience a life-threatening release of calcium from the sarcoplasmic reticulum when given anesthetics or muscle relaxants [225]. The mutation is a substitution of an arginine to histidine or cysteine in the cytoplasmic III–IV linker that appears to lower the depolarization threshold needed to induce the ryanodine receptor to release calcium from the sarcoplasmic reticulum stores [226].

The Ca_v1 family is important in cardiac and smooth muscle function. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are involved in pacemaking activity for the heart by controlling rhythmic entry of calcium in the sinoatrial and atrioventricular nodes. They also have roles in neurons along with the classical neuronal Ca_v2 family in regulating synaptic activity and gene expression. Unlike the predominantly axonal distribution of Ca_v2 channels, $\text{Ca}_v1.2$ – 1.4 have a more somatodendritic distribution in neurons. Ca_v1 channels possess a binding site for calmodulin, a calcium binding protein that can activate many signaling pathways in a calcium-dependent manner. Synaptic activity that leads to Ca_v1 channel opening can initiate nuclear signaling and activation of transcription factors such as cAMP response element binding (CREB) protein, leading to changes in gene transcription. Ca_v1 channels are able to both recruit and

activate calmodulin by providing a calmodulin binding site as well as a calcium ion source from the open channel. Calcium-bound calmodulin is capable of activating a number of kinase pathways, including ones that phosphorylate CREB, encouraging its nuclear entry and activity as a regulator of transcription [227].

$\text{Ca}_v1.4$, the most recently cloned of the Ca_v1 family, is the least understood, but a channelopathy, incomplete congenital stationary night blindness (CSNB2), caused by mutations in $\text{Ca}_v1.4$ has illuminated one of the roles of this channel. CSNB2 presents itself with a combination of symptoms such as myopia, nystagmus, reduced visual acuity, or night blindness. Over 40 mutations in $\text{Ca}_v1.4$, ranging from amino acid substitutions to truncations, have been identified in CSNB2 patients [228]. It is not clear how these particular mutations of $\text{Ca}_v1.4$ compromise channel function. $\text{Ca}_v1.4$ is ideally suited for controlling tonic neurotransmitter release because it has a rather low threshold for activation at around -40 mV and inactivates slowly. Moreover, $\text{Ca}_v1.4$ is expressed in the retina, and electroretinograms of CSNB2 patients indicate a defect in the tonic neurotransmission that occurs between photoreceptors and second-order neurons [229].

The most varied and clinically useful Ca^{2+} channel pharmacological agents are those that block Ca_v1 channels to treat hypertension, angina, and certain arrhythmias [230]. The actions of dihydropyridines on Ca_v1 channels have been studied since the 1980s and their influences on the channel are complex. The ability of dihydropyridines to interact with Ca_v1 channels is state dependent; dihydropyridines are inferior blockers when Ca_v1 Ca^{2+} channels are closed during hyperpolarizing conditions. There is also evidence that dihydropyridines promote the Ca^{2+} channel to be in an inactive state, reducing the channel's current [231]. The sites of dihydropyridine interaction with Ca_v1 have been isolated using mutagenesis studies, and a key conserved site is a threonine in S5 of domain III in $\text{Ca}_v1.2$ [232]. Moreover, a number of sites in domains III and IV also contribute to Ca_v1 's dihydropyridine sensitivity [233, 234].

Like cardiac and skeletal muscle, vascular smooth muscle utilizes voltage-gated Ca^{2+} channels for regulating intracellular calcium levels that are responsible for muscular contraction. Using the dihydropyridine group of drugs has been clinically useful to treat hypertension by blocking Ca_v1 [235]. Reducing Ca_v1 channel activity reduces calcium entry into vascular smooth muscle and consequently reduces vascular pressure.

14.4.5.3 Ca_v2 Family. The members of the Ca_v2 family are expressed in a range of organs such as the pancreas, heart, and testis. However, their widespread presence in the central nervous system and their importance in neuronal function have been the overriding focus of Ca_v2 channel studies. The three Ca_v2 channel types have different pharmacological sensitivity profiles and specialized roles, but a common trait among them is their regulation of neurotransmitter release. Located at the presynaptic end of an axon, Ca_v2 channels open and allow calcium to enter the axonal terminal when an action potential pulse reaches the end of its path along an axon. Calcium ions that enter through Ca^{2+} channels and the channels themselves are key players in second-messenger pathways leading to presynaptic release of neurotransmitters [236]. $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ contain a synaptic protein interaction motif in the II–III intracellular loop that binds with various proteins that are part of the neurotransmitter release machinery such as synaptotagmin I, syntaxin I, and SNAP-25 [237]. This

voltage-gated Ca^{2+} channel involvement in linking electrical activity with exocytic release is also utilized in other physiological contexts such as hormone release [238].

Unlike the Ca_v1 family, individual Ca_v2 α subunits have specific blockers. The best known pharmacological modulators of the Ca_v2 family are peptides derived from the venom of predatory animals. Comprised of 20–30 amino acids, these peptides can be rather specific, capable of binding to a particular Ca_v2 subunit at nanomolar concentrations.

The most specific blocker of $\text{Ca}_v2.1$ is the 48-amino-acid ω -agatoxin isolated from the American funnel spider *Agelenopsis aperta*. However, it also blocks $\text{Ca}_v2.2$ with low affinity, as well as $\text{Ca}_v2.3$. Blockade by ω -agatoxin is irreversible and likely interferes with the $\text{Ca}_v2.1$ voltage sensor. The S3–S4 region of DIV is the major site where ω -agatoxin interacts with $\text{Ca}_v2.1$ [239]. The P- and Q-type calcium currents arise from different splice variants of $\text{Ca}_v2.1$. The Q-type $\text{Ca}_v2.1$ splice variant has an addition of an asparagine and proline in the agatoxin interaction region, resulting in a lower agatoxin binding affinity.

The venom from the sea snail *Conus geographus* contains a 27-amino-acid peptide ω -conotoxin GVIA that is a selective, irreversible blocker of $\text{Ca}_v2.2$ N-type channels. ω -Conotoxin GVIA blocks $\text{Ca}_v2.2$ by physically occluding the pore [240]. Other selective N-type blocking peptides are ω -conotoxin MVIIA and MVIIIC from the *Conus magus* snail and the ω -conotoxin CVID isolated from *Conus catus*.

The therapeutic and commercial success of Ca_v1 channel blockers has inspired the possibility that specific Ca_v2 channel blockers have therapeutic potential as well. The most promising application is to use the Ca_v2 channel blockers for pain treatment. The impetus for finding pharmacological modulators of Ca_v2 channels is that the channels are highly expressed in the superficial layer of the dorsal horn of the spinal cord, an area where nociceptive signaling is transmitted. It is hoped that blocking Ca_v2 channels will block mediators of nociceptive signaling such as substance P [241]. The promise of this strategy is supported by $\text{Ca}_v2.2$ knockout mice having lowered sensitivity to neuropathic and inflammatory pain, and current μ -opioid receptor agonists used for pain relief, such as morphine, partially block Ca_v2 channels [242, 243]. Ziconotide, a compound derived from ω -conotoxin MVIIA, has been approved for clinical use by the Food and Drug Administration (FDA) and the European Commission for treating severe chronic pain. Currently, ziconotide can only be administered intrathecally and there are some cases of severe side effects such as unruly behavior and hypotension [244]. More toxin-derived Ca_v2 channel blockers are in various stages of development for pain treatment [245]. In addition, small organic molecules that specifically target Ca_v2 have the advantage of being orally administered and are also being developed and reviewed for pain treatment [246].

Until recently, there were no known pharmacologic blockers of $\text{Ca}_v2.3$ Ca^{2+} channels, but it is now known that these channels are blocked by SNX-482, a compound isolated from the venom of the tarantula *Hysteroecrates gigas* [247]. SNX-482 also partially inhibits Ca_v1 channels with low affinity, but SNX-482 is the best $\text{Ca}_v2.3$ blocker to date [248].

14.4.5.4 Ca_v3 Family. The Ca_v3 family is the least understood among the voltage-gated channels, but its members are known to be expressed in many tissues. Opening

at potentials near the resting membrane potential, Ca_v3 channels can influence the membrane potential of the cell at rest. There is also evidence that Ca_v3 channels affect rhythmic neuronal firing.

Despite years of intense effort, there are still no specific and effective pharmacological blockers of Ca_v3 channels. As a result, studies of Ca_v3 have been hindered, but there is growing evidence for functional roles of Ca_v3 channels and the potential benefits of developing specific Ca_v3 pharmacological targets.

Like $\text{Ca}_v2.2$, $\text{Ca}_v3.2$ and $\text{Ca}_v3.3$ are expressed in the dorsal root ganglion neurons of the superficial lamina. Given Ca_v3 's low activation threshold kinetics, blocking these channels can reduce the overall excitability of a neuron. A number of pain models suggest that an increase in excitability of nociceptive neurons contributes to pain perception. Blocking Ca_v3 neurons could hamper these neurons' excitability and subsequently suppress nociceptive signaling [245].

14.4.5.5 Auxiliary Subunit Modulators. Given the substantial functional modifications that auxiliary subunits can have on voltage-gated Ca^{2+} channel properties, drugs that bind to these Ca^{2+} channel auxiliary subunits may also lead to alterations in Ca^{2+} channel function. One such example is the anticonvulsant drug gabapentin. Gabapentin interacts with a number of proteins, including $\alpha_2\delta_1$ [249]. Both the α_2 and δ subunits are required for gabapentin binding, but it is not known how this binding alters voltage-gated Ca^{2+} channel function. The general effect of gabapentin on voltage-gated Ca^{2+} channels is a reduction in macroscopic current. Besides the anticonvulsant properties of gabapentin, it has also been found to relieve neuropathic pain. Gabapentin's analgesic properties may be attributable to its block of $\text{Ca}_v2.2$ current in the dorsal root ganglia.

14.5 OTHER VOLTAGE-GATED CHANNELS

The superfamily of voltage-gated cation channels contains several other important channel types, including the cyclic nucleotide-gated (CNG) channels, the hyperpolarization-activated channels (HCN), and the transient receptor potential (TRP) channels. CNG channels are nonselective cation channels and are crucial for the receptor-mediated transduction of visual and olfactory information through cyclic nucleotide second-messenger pathways [250]. HCN channels are nonselective, gated by cyclic nucleotides, and are opened at voltages hyperpolarized to the resting potential. They play a role in pacemaker activity of neurons and of the heart [251]. TRP channels are nonselective and only weakly voltage sensitive. They are more distantly related to Kv channels than the HCN or CNG families. These channels play a role in a variety of sensory systems, including nociception, thermosensation, and taste sensation [252].

Other unrelated classes of voltage-gated ion channels include voltage-gated proton channels and voltage-gated chloride channels. The voltage-gated proton channel is an essential part of the oxidative burst of phagocytic cells of the immune system, providing a cationic shunt for the large, anionic superoxide flux generated by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [253]. This channel has not yet been cloned. Voltage-gated chloride channels, or ClC channels, are a subset of anion channels that is entirely

unrelated structurally to cation channels. These channels modulate muscle cell excitability, endosomal and vesicular acidification, epithelial transport, bone resorption, and cell swelling [254].

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15

NEUROPEPTIDES

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15.1	Neuropeptide Concept	670
15.2	Identification of Neuropeptides	671
15.3	Isolation and Characterization of Neuropeptides	674
15.4	Peptidomics	675
15.5	Neuropeptide Receptor Agonists and Antagonists	675
15.6	Electrophysiological Techniques	675
15.6.1	Neurohormone, Neurotransmitter, or Neuromodulator?	675
15.6.1.1	Neurohormone	675
15.6.1.2	Neurotransmitters	676
15.6.1.3	Neuromodulators	676
15.7	Perfusion and Tissue Culture Studies	677
15.8	Behavioral Techniques	677
15.9	Genetic Manipulations	678
15.9.1	Transgenic Animals	678
15.9.2	Gene Targeting and Knockout Mice	678
15.9.3	Genomics	678
15.9.4	Site-Directed Mutagenesis	679
15.10	Integrated Physiological, Peptidomic, and Genomic Techniques	679
15.11	Evolution of Neuropeptides	679
15.11.1	Conservation of Structure	679
15.11.2	Gene Duplication and Neuropeptide Families	680
15.11.2.1	Gene Duplication in the Opioid Family	680
15.11.2.2	Gene Duplication in the Neurohypophyseal Family	682
15.11.2.3	Duplication and Divergence in the NPY Family	684
15.11.2.4	Gene Splicing	685
15.12	Organization of Neuropeptide Genes	685
15.12.1	Regulation of Gene Expression	685
15.13	Biosynthesis and Processing of Neuropeptides	685
15.13.1	Preprohormones	686
15.13.2	Prohormones and Precursor Processing	686
15.13.2.1	Role of Prohormones	686
15.13.3	Tissue-Specific Processing	687

15.14	Inactivation of Neuropeptides	687
15.14.1	Intracellular Degradation	687
15.14.2	Extracellular Inactivation	687
15.15	Neuropeptide Receptors	688
15.15.1	Receptor Downregulation and Desensitization	688
15.15.2	Receptor Upregulation and Sensitization	689
15.15.3	Isoreceptors and Receptor Subtypes	689
15.15.3.1	Arginine Vasopressin Receptors	689
15.15.3.2	Neuropeptide Y Receptor System	689
15.15.3.3	Tachykinin Receptors	689
15.15.3.4	Melanocortin Receptors	690
15.16	Neuropeptide Receptors and Second-Messenger Systems	690
15.16.1	G-Protein-Coupled Receptors	690
15.16.2	Phospholipase–Phosphatidylinositol Linked Messengers	690
15.16.3	Guanylate Cyclase (cGMP) Receptors	691
15.16.4	Tyrosine Kinase–Coupled Receptors	691
15.16.5	Cytokine Receptors	691
15.17	Hypothalamic Control of the Pituitary Gland	691
15.17.1	Hypothalamic Release-Stimulating and Release-Inhibiting Neuropeptides	691
15.17.1.1	Control of Prolactin Release	691
15.17.2	Hypothalamic–Pituitary Pathways	692
15.17.3	Additional Pathways for Neuropeptide Delivery	692
15.18	Blood–Brain Barrier	692
15.19	Administration of Neuropeptides	693
15.20	Time- and Tissue-Sensitive Responses to Neuropeptides	693
15.21	Neuropeptide Redundancy	694
15.22	Stress and Neuroendocrine Response	694
15.23	Summary	695
	References	696

15.1 NEUROPEPTIDE CONCEPT

The neuroendocrine system integrates the regulatory functions of the nervous and endocrine systems, involving the neurosecretions of hypothalamic neurons controlling the activity of the anterior pituitary gland. These interactions are notable for the elaborate systems of feedback controls, chiefly negative. Since the late 1960s and early 1970s, when it was shown that the pituitary and hypothalamic hormones are peptides, that the endorphins and enkephalins are peptides, and that the gut peptides—such as cholecystokinin (CCK), vasoactive intestinal polypeptide (VIP), and somatostatin—are also produced in the brain, interest and investigation into the actions of peptides have grown exponentially.

What makes a peptide a neuropeptide? The demonstration that brain peptides have direct effects on neurons involved in behavior was first shown by De Wied [1] and Kastin et al. [2]. The dissociation of the endocrine and neural effects of adrenocorticotrophic hormone (ACTH) using fragments devoid of adrenocorticotrophic action [3–6] led to the *neuropeptide concept*: that peptides produced in the brain

and gut have direct effect on central and peripheral neurons. This novel idea took many years to be accepted [7]. Today we are aware that neuropeptides affect nonneural tissues and organs as well as neurons and they integrate almost every system of the body: reproduction; growth; water and salt metabolism; temperature control; food and water intake; cardiovascular, respiratory, and gastrointestinal control; and affective states. When we add to this list their participation in autonomic responses, nerve development, and regeneration and their increasing involvement with food intake and body weight regulation [8], their importance becomes self-evident.

New and ever more sophisticated methodologies have enabled the better understanding of the actions, relationships, and regulatory actions of these molecules. The number of neuropeptides identified grows almost daily, and peptides with neurogenic activities have been isolated from many nonendocrine sources. They are found in the brain and spinal cord, sensory neurons of the spinal ganglia that contain substance P (SP), somatostatin (ST), VIP, and CCK, among other neuropeptides, and almost all peripheral organs where they may act as autocrine, paracrine, or endocrine factors. They are present in sources as disparate as frog skin, shark mouth, the invertebrates, and plants, most of which affect mammalian tissues as well as their host tissues. Table 15.1 indicates the source of some of the most important neuropeptides.

The coexistence of neuropeptides with the classical neurotransmitters, such as norepinephrine and acetylcholine or γ -aminobutyric acid (GABA), extends the dispersion of many neuropeptides to central, peripheral, and autonomic nerve terminals, and the presence of two or more neuropeptides in the same nerve terminal indicates the complexity of their possible synergistic/antagonistic actions. Neuropeptides have been reported to coexist with transduction molecules, suggesting that peptides are not randomly expressed with regard to their signal transduction molecules.

Topics to be discussed in this chapter will include the identification and processing of neuropeptides, the evolution of neuropeptide families together with the evolution of their complex receptor subtypes, the importance of time-sensitive responses to neuropeptides, especially in development and regeneration, and the apparent redundancy of many neuropeptides as evidenced by “knockout” animals. Information derived from new methodologies such as genomics and proteomics brings our understanding of neuropeptides to a genomic and even molecular level.

15.2 IDENTIFICATION OF NEUROPEPTIDES

In this section we discuss some of the techniques used to identify neuropeptides:

1. *Bioassay* This is the earliest technique for the functional identification of a hormone. It determines the effect of administration of that hormone to an animal from which the endogenous source of that hormone has been removed. Removal of the thyroid gland results in severe metabolic disturbances that can be returned to normal through the administration of thyroxine (T_4). The concentration of a hormone in tissue, plasma, or urine can also be quantified, but most in vivo bioassays are not sensitive enough to measure normal or below-normal levels of the hormone.

TABLE 15.1 Mammalian Neuropeptides Classified According to Principle Sources and Other Sources

Principal Sources	Other Sources
Hypothalamic Peptides	
Agouti-related protein	
β -Endorphin	
CART ^a	Pituitary, striatum, adrenal medulla
Corticotropin-releasing hormone	Pituitary, median eminence, brain, spinal cord
Galanin	Locus ceruleus
Gonadotropin-releasing hormone	Placenta, gonads?
Growth hormone-releasing hormone	Placenta
Melanin-concentrating hormone	
Orexin/hypocretin	
Oxytocin ^b	Posterior pituitary, brain, spinal cord
Pituitary adenylate cyclase-activating polypeptide	Thalamus, hippocampus, spinal cord
Proopiomelanocortin	Pituitary
Somatostatin	Brain, spinal cord, gut, salivary glands, excretory system
Thyrotropin-releasing hormone	Brain, spinal cord, gut, pancreas
Tyr-MIF-1	Cerebral cortex
Vasopressin ^b	Posterior pituitary, brain, spinal cord, peripheral nerves
Anterior Pituitary Peptides	
Adrenocorticotropin	Median eminence, brain, spinal cord, placenta, gut, lymphocytes
β -Endorphin	Median eminence, placenta, gut
Follicle-stimulating hormone	Median eminence, placenta
Growth hormone	Median eminence, placenta
Luteinizing hormone	Median eminence, placenta
Melanocyte-stimulating hormone	Median eminence, brain, spinal cord, peripheral nerves
Prolactin	Median eminence, brain, spinal cord, placenta
Thyroid-stimulating hormone	Median eminence, placenta
Brain and Spinal Cord Peptides	
δ Sleep-inducing peptide	
Dynorphins	
Endorphins	Pituitary
Met- and Leu-enkephalin	Myenteric neurons, peripheral nerves
Neuropeptide glutamine (<i>E</i>)isoleucine-(1)	
Neurotensin	Cardiovascular system, gastrointestinal tract
Nociceptin/orphanin	Brain stem, trigeminal ganglion, central nervous system?
Secretoneurin	

TABLE 15.1 (Continued)

Principal Sources	Other Sources
Major Gut and Pancreatic Peptides	
Ghrelin	Stomach, intestine, placenta, pituitary, hypothalamus?
Calcitonin gene-related peptide, gastrointestinal tract	Brain, spinal cord, neuromuscular junctions, thyroid
Cholecystokinin, intestine	Brain, pituitary, adrenal medulla, peripheral nerves
Galanin, intestine	Brain, spinal cord, almost all peripheral systems and organs
Gastrin, gastric antrum	Pituitary, spermatozoa

Source: From [9], with permission from The MIT Press.

^aCocaine- and amphetamine-regulated transcript.

^bProduced in hypothalamus but stored in neurohypophysis and called neurohypophyseal peptides.

2. *Cytochemical Assay* This technique, developed by Chayen and Bitensky [10], is a more sensitive technique than the bioassay and is based on the development of a colored precipitate formed as a result of a hormone-dependent intracellular reaction. The precipitate is analyzed by microspectrometry and microdensitometry.
3. *Radioimmunoassay (RIA)* The RIA was developed by Berson and Yalow in 1960 and is less sensitive than the cytochemical assay, but it is less demanding. It can measure femtomoles of neuropeptides and is the most convenient and accurate method of determining neuropeptide concentrations, especially in characterizing the rhythms of the release of many of the hypothalamic and pituitary hormones.
4. *Immunocytochemistry* This technique permits the anatomical localization of neuropeptides through their immunoreactivity as detected by specific antisera. Classically this method has demonstrated the pathways of monoamines in the central nervous system (CNS) [11]. A major disadvantage of this technique is that the antibodies may recognize biologically inactive parts of the peptide or bind to the degraded portion of the peptide. Consequently, it is usual to refer to the reactivity as “-like,” as in growth hormone-like immunoreactivity.
5. *Immediate Early Genes* These are expressed early and transiently (within an hour) in active tissues, permitting the location of cellular activity through immunocytochemical techniques that locate the immediate early genes. The best studied immediate early genes are *c-fos* and *c-jun*. When this technique is combined with pathway-tracing methods, it provides a powerful tool for defining active neuropeptide pathways [12].
6. *Autoradiography* This consists of the administration of a radioactive neuropeptide and the subsequent incubation of tissue slices or cell cultures to permit the visualization of the binding site. Other labeling techniques are used, such as labeling with colloidal gold and subsequent identification through electron

microscopy [13, 14]. *Neuropeptide receptors* can be selectively labeled and visualized microscopically on autoradiograms (microscopic receptor mapping). In vivo receptor mapping has several drawbacks [15]. In vitro visualization of receptors is much more sensitive as the visualization is amplified through image-intensified fluorescence microscopy and fluorophores, the most commonly used being fluorescein and rhodamine derivatives [16].

7. *In Situ Hybridization and Histochemistry* This is used more often because the visualization of the receptor messenger RNAs (mRNAs) permits the identification of the cells that synthesize the receptors. The development of highly selective agonists and antagonists, together with quantitative receptor autoradiography in vivo and in vitro, has revealed complex pharmacological responses and specific receptor subtypes [17]. *Histochemistry* is used to study the regulation of selected mRNA species in single neurons and is based on the ability of radiolabeled DNA that is complementary to a specific mRNA to bind to specific mRNA in tissue sections. Either an oligonucleotide or an RNA probe is usually selected. Immunohistochemistry is usually performed before in situ hybridization to localize both the hybridization signal and an immunohistochemical label within the same cell [18].

Other techniques that measure neuropeptide release include in vivo sampling and microdialysis, antibody microprobes, in vivo perfusion methods, reversed-phase high-performance liquid chromatography (HPCL) [19], and the cell blot assay [20].

15.3 ISOLATION AND CHARACTERIZATION OF NEUROPEPTIDES

Many methods have been used for the isolation and characterization of neuropeptides, including biological and chemical assays, peptide purification, peptide and nucleotide determination, and molecular biotechnology. Peptides isolated on the basis of one biological function often have many other functions. Peptides isolated on the basis of their chemical characteristics frequently have no known biological function, because biological activity often depends on posttranslational modifications. Many synthetic neuropeptides, manufactured on the basis of their amino acid sequence in the precursor hormone, may have potent biological effects yet cannot be demonstrated in physiological systems. However, some synthetic analogs have more powerful and longer lasting effects than the endogenous neuropeptide, an outcome of considerable clinical significance.

Peptides can be separated by chromatography and capillary electrophoresis and detection and by absorbance, fluorescence, electrochemistry, and immunoassay techniques [21]. Some frequently used methods of peptide isolation and structure analysis include fast atom bombardment spectrometry [22], size exclusion HPLC linked to electrospray ionization mass spectrometry [23], and matrix-assisted laser desorption ionization time-of-flight mass spectrometry [24]. These techniques, when combined with two-step liquid chromatography, provide another rapid and reliable method for the identification of bioactive molecules, as recently described for five new bradykinin-potentiating peptides [25].

15.4 PEPTIDOMICS

The term peptidomics refers to techniques that permit the quantitative determination of the peptide content of whole cells. New peptides are being discovered using single-cell mass spectrometry, which permits the measurement of molecular weights of peptides and proteins from single-cell samples [26]. A technique that includes the virtual visualization and identification of the peptides as two- and three-dimensional image maps is a powerful method for investigating endogenous peptides and their posttranslational modifications in complex tissues such as the brain [27, 28]. Recently, an approach termed *combinatorial peptidomics* was introduced. This is based on the selective crosslinking of peptides to a bead surface specifically reactive for a peptide side chain such as sulfhydryl or hydroxy [29].

15.5 NEUROPEPTIDE RECEPTOR AGONISTS AND ANTAGONISTS

The development of peptide receptor antagonists that selectively block the action of the peptide through binding to its receptor is an invaluable tool in investigating the function of those peptides for which receptor antagonists have been developed. The opiate antagonist naloxone is a specific narcotic antagonist and has been used extensively to study the specificity of opiate action. Synthetic antagonists to SP and other members of the tachykinin family have resulted in the identification of several receptor types and subtypes, often with quite different properties.

15.6 ELECTROPHYSIOLOGICAL TECHNIQUES

Neuropeptides can modulate the ion permeability of the neuronal membrane [30]. Many neuropeptides act as neurotransmitters, neuromodulators, or both, and these functions can best be investigated by electrophysiological methods. A common technique in the CNS utilizes chronically implanted microelectrodes that record single cells in conscious animals. Neuropeptides may be administered directly onto individual cells by microiontophoresis or pressure ejection and the response of the target cell recorded. These methods have been able to distinguish whether the applied neuropeptide is acting as a neurotransmitter or neuromodulator depending on the change in membrane potential of the responding cell [31].

15.6.1 Neurohormone, Neurotransmitter, or Neuromodulator?

15.6.1.1 Neurohormone. A neurohormone is secreted into the circulation and binds to a specific receptor on the target cell membrane. This initiates a cascade of metabolic reactions, usually through G-coupled proteins and cyclic adenosine monophosphate (cAMP). *Classical neurotransmitters* such as acetylcholine (ACh) epinephrine, dopamine, and GABA are synthesized in nerve terminals and stored in small, clear vesicles. Synthesis is rapid and there is little chance of neurotransmitter depletion. In contrast, synthesis of neuropeptides is slow and they are stored in large dense-core vesicles (LDCVs) that are moved by axonal transport to nerve terminals. Neuroendocrine cells may respond to either or both electrical stimulation and

hormonal stimulation. Hormonal stimulation induces secretion in neuroendocrine cells via binding of the hormone to the membrane-bound receptor, initiating the cascade of second-messenger reactions and the release of cytosolic Ca^{2+} . *Exocytosis*, a calcium-dependent function, entails the fusion of the membrane of the LDCVs with the presynaptic membrane. Details of this process are described by Nicholls [32] and Burgoyne and Morgan [33]. The contents of the LDCVs are spilled into the extracellular space and the vesicle membranes recycled.

Neuroendocrine cells respond to an electrical stimulus through voltage-gated sodium, calcium, and potassium channels that mediate the all-or-nothing potentials associated with the rise in $[\text{Ca}^{2+}]_i$. Four major types of calcium channels have been identified, and after a rise in cytosolic free Ca^{2+} , neuroendocrine cells display a wide repertoire of electrical responses to secretagogues [34].

15.6.1.2 Neurotransmitters. Neuropeptides acting as neurotransmitters are characterized by latencies on the order of hours rather than milliseconds. Higher frequencies are usually required for a neuropeptide, secreted from a neuroendocrine cell and acting as a neurotransmitter, to evoke a response, which then is slower and longer lasting [35]. As with the classical neurotransmitters, neuropeptide release is Ca^{2+} dependent, but there is no evidence for organization of the dense-core neuropeptide-containing vesicles at the presynaptic membrane. Again, unlike the classical neurotransmitters, there does not appear to be any specific inactivating mechanism: Inactivation depends on nonspecific proteases and peptidases. SP is the typical excitatory neuropeptide transmitter, producing slow noncholinergic excitatory postsynaptic potentials (EPSPs) on spinal motor neurons and on neurons in sympathetic ganglia. Depletion of SP by capsaicin abolishes these potentials. Similar experiments with gonadotropin-releasing hormone (GnRH) showed it to be a neurotransmitter in frog sympathetic ganglia where it coexists with ACh. GnRH produces a late, slow EPSP, different from the rapid ACh-evoked potentials [36]. SP and GnRH are secreted from presynaptic nerve endings in response to Ca^{2+} influx and may either hyperpolarize or depolarize the postsynaptic membrane. A single, above-threshold impulse is sufficient to cause a response.

15.6.1.3 Neuromodulators. Neuromodulators fine tune synaptic and nonsynaptic processes by altering the effects of the classical neurotransmitters with which they coexist in nerve terminals. They act more slowly than the classical neurotransmitters and their effects are longer lasting as they affect G-protein-linked receptors and subsequent second-messenger systems. When a neuropeptide is acting as a *neuromodulator*, it also is secreted in response to Ca^{2+} influx, but it is not capable of evoking an excitatory (EPSP) or inhibitory postsynaptic potential (IPSP). Rather it facilitates an EPSP or exacerbates an IPSP evoked by a neurotransmitter. The modulatory action of thyroid-stimulating hormone (TSH) on the discharge patterns of neurons in the brain stem is clearly demonstrated by Dekin et al. [37]. Neuromodulator release is dependent upon the duration, frequency, and pattern of nerve stimulation. Neuromodulators are effective in very low concentrations (picomolar), but their biological activity may depend upon the form of the processed neuropeptide; for example, somatostatin 28, somatostatin 14, and somatostatin 12 may differ considerably in their potencies. No specific inactivating mechanisms are known.

A neuropeptide may act as a neurohormone at some sites, a neurotransmitter at others, and a neuromodulator at still other sites. For example, hypothalamic neurons release vasopressin (VP) into the posterior pituitary, where it is stored and released into the circulation as a neurohormone, whereas other nerve endings of hypothalamic neurons containing VP may terminate in various brain areas where it acts as either a neurotransmitter or a neuromodulator. In the gut and in both male and female reproductive systems vasoactive intestinal polypeptide (VIP) acts as a neurotransmitter, evoking widespread relaxant activity and increasing blood flow to the sex organs. VIP coexists with ACh in both central and peripheral cholinergic synapses where it modulates cholinergic activity by a postsynaptic mechanism. In the frog neuromuscular junction, VIP acts as a modulator but by a different mechanism: It increases the release of ACh but has no direct postsynaptic effect [38]. Similarly, SP, an excitatory neurotransmitter in spinal motoneurons, is a neuromodulator in sympathetic ganglia where it facilitates both nerve-stimulated EPSPs and the depolarization caused by local application of ACh.

Interestingly, many *insect neuropeptides* also appear to be multifunctional in that they may act as neuromodulators and/or neurohormones, regulating homeostasis, the organization of behaviors, and the initiation and coordination of development and neuromuscular activity. They lend themselves favorably to the study of neuropeptide function due to the ready availability of molecular genetics methods [39].

Neuropeptides may be *colocalized* with other neuropeptides or with the classical neurotransmitters, such as ACh, norepinephrine, dopamine, serotonin, or GABA. In some neurons, two or more neuropeptides may be present in the same secretory granule and, depending on the processing of the prohormone, lend immense flexibility to the regulation and modification of synaptic events [40].

15.7 PERFUSION AND TISSUE CULTURE STUDIES

Many different *in vitro* perfusion preparations are used, ranging from the isolated pituitary gland to chunks of tissue such as hypothalamic–neurohypophyseal explants, in which much of the neural and vascular connections can be maintained. Brain perfusion studies have identified separate and distinct transport systems of neuropeptides across the blood–brain barrier (BBB) [41]. Penetration of neuropeptides across the BBB is discussed in Section 15.18.

Fetal spinal cord slices or dissociated spinal and sensory neurons can be propagated in a liquid culture medium or in a semisolid agar medium and the effect of neuropeptides on neurite growth determined [42, 43]. A more sensitive assay uses the three-chambered tissue culture system [44, 45].

15.8 BEHAVIORAL TECHNIQUES

The pioneering studies by De Wied and his group [46, 47] and Kastin and his colleagues [48] clearly demonstrated that ACTH and its noncorticotropic peptide fragments, including melanocyte-stimulating hormone (MSH), have potent effects on adaptive behavior in rats. Behavioral tests in humans include tests of visual

discrimination, attention, and memory. Attentional and processing abilities may be enhanced by ACTH or MSH fragments [49]. There is some correlation between changes in brain peptides and Alzheimer's disease [50].

15.9 GENETIC MANIPULATIONS

15.9.1 Transgenic Animals

The generation of transgenic mice has become a powerful device for focusing on the mechanisms of gene regulation, gene promoter characteristics, enhancer sequences, and tissue-specific expression. Transgenesis applies to the introduction of new genes into mammalian organisms and the resulting animals are referred to as transgenic animals. The techniques of producing transgenic mice are described in detail by Evans et al. [51]. The overall physiological consequences of a specific, artificial mutation can be investigated in the intact animal and the effects of an over- or underproduction of a specific neuropeptide determined. This is a powerful tool for focusing on the mechanisms of gene regulation. In a mouse model that expresses a transgene that causes an overproduction of corticotropin-releasing hormone (CRH) the animals exhibit endocrine abnormalities involving the hypothalamic–pituitary–adrenal (HPA) axis and serve as a genetic model for chronic stress [52, 53].

15.9.2 Gene Targeting and Knockout Mice

An important research tool is gene targeting, a procedure in which a single gene is selected and inactivated or “knocked out.” The inactivated (null) gene is injected into early mouse embryos and replaces the normal gene in the mouse chromosome. Subsequent generations of deficient mice have an inheritable, complete loss of function in all tissues at all stages of development that were affected by the normal gene. The specific role of the knocked-out gene can then be determined by observing the physiological and structural changes resulting from the absence of the normal gene. A large number of neuropeptide genes have been tested for their ability to target expression to the hypothalamus and pituitary in transgenic mice [54]. A CRH-deficient mouse has deficiencies in stress responses, diurnal rhythms, and feeding behavior. Some of the problems involved in producing the null CRH gene are discussed by Muglia et al. [55]. Neuropeptide Y (NPY) knockout mice and mice and rats overexpressing the NPY gene indicate that the stress-related behaviors and regulation of involuntary alcohol intake are perhaps clinically the most important functions of central NPY [56]. Similarly, the physiological actions of peptides of the calcitonin family have been recently defined through gene knockout and overexpression strategies [57].

15.9.3 Genomics

Modern genetic techniques, such as genomics, are being used to probe the molecular basis of behavior in animals. A classic example is that of the prairie vole, an animal that is monogamous, and the closely related promiscuous meadow vole. This trait is governed by the neuropeptide VP and the DNA sequence for VP is the same in both

species. Similarly the sequence for the receptor gene is the same. However, the prairie voles have far more VP receptors. The difference lies in the longer regulatory region at the beginning of the receptor gene in the prairie vole. When the prairie vole's VP receptor gene, together with its regulatory region, is placed into meadow vole embryos, the resulting adult males changed their promiscuous behavior and remained faithful to their mates [58].

15.9.4 Site-Directed Mutagenesis

With the ability to create almost any desired mutation in a given DNA sequence, site-directed mutagenesis permits the introduction of a selected mutation into a specific location. This approach is invaluable for the study of neuropeptides, their precursors, and the precise sequence(s) responsible for such processes as receptor binding [59].

15.10 INTEGRATED PHYSIOLOGICAL, PEPTIDOMIC, AND GENOMIC TECHNIQUES

Many neuropeptides affect a great many physiological processes. Neuropeptide Y and galanin are two hypothalamic neuropeptides that affect a variety of physiological reactions related to nutrient and energy homeostasis. To study these interactions, endocrine, metabolic, behavioral, and neural techniques are required [60]. The complex physiological responses to the stress-evoked hormones [CRH, ACTH, VP, oxytocin (OT), endorphins, and prolactin] are interpreted through measurements of cardiac and pressor changes, alterations in reproductive endocrinology and behavior, analgesia and locomotor activity, and alterations in the immune system. How genes are regulated is of importance equal to that of gene mutations and polymorphisms in differential susceptibility to disease. Information derived from postgenomic bioinformatic and transgenic approaches, together with the mass of information available on the genetic linkage of noncoding polymorphisms, can help us understand how inappropriate regulation of neuropeptide gene expression can lead to human disease [61]. The challenge is to bring order into the mass of data and discover the pattern by which neuropeptides regulate physiological processes.

15.11 EVOLUTION OF NEUROPEPTIDES

15.11.1 Conservation of Structure

The structure of neuropeptides has been strongly conserved during evolution and must have evolved together with the necessary components of the signaling system, such as receptors, processing enzymes, and inactivation mechanisms. Once this complex integrated system had evolved in simple organisms, it probably would have remained intact in more complex animals, conserving the more active portion of the molecule. For example, the mammalian insulins are very highly conserved with only one or two sequence differences between porcine, bovine, and human insulin, suggesting that almost all the insulin structure is essential for its function. In contrast, there is little homology between porcine and rat relaxin, a peptide hormone

produced during pregnancy, indicating considerable divergence between these two species. The hypothalamic decapeptide GnRH, which stimulates the pituitary to produce the gonadotropic hormones that regulate reproduction and sexual behavior, plays a pivotal role in all vertebrates and in some invertebrates and protochordates [62–64] and therefore is highly conserved. Through gene duplication two and even three forms of GnRH have evolved [65].

Conservation of structure does not mean retention of initial function or inability to gain new functions. Prolactin (PRL), one of the oldest peptide hormones, has changed its function radically throughout evolution. In all species, from fish to humans, PRL is involved in water and salt metabolism; in birds it is vital for crop milk production; in mammalian females it is responsible for lactation; and in mammalian males, including humans, PRL is involved in spermatogenesis, testosterone synthesis, and male libido.

15.11.2 Gene Duplication and Neuropeptide Families

Neuropeptides form families of closely related peptides in which the biologically active part of the molecule is closely conserved (Table 15.2). Our understanding of the evolution of neuropeptide families is incomplete, but analyses of the amino acid sequences and the identification of the whole peptidome of a cell or tissue (peptidomics) lead to the concept that these neuropeptide families have been generated through successive events of gene and exon duplication within a common ancestral neuropeptide. This process temporarily frees one part of the genome from selective pressure and permits a small error rate of mutation. New receptors and processing enzymes probably also evolved from existing receptors through gene duplication and show familial relationships as required by their neuropeptide ligands and substrates respectively. PRL, one of the oldest of the neuropeptides, belongs to the growth hormone family, which includes the insulin-like family, the enkephalins, the gut hormones, and the tachykinins (SP, neurokinin A, neurokinin B, eladoin, and physalaemin), all of which share the amino acid sequence of SP. A fine review of the role of mutations in the evolution of vertebrate neuropeptides can be found in Holmgren and Jensen [66].

15.11.2.1 Gene Duplication in the Opioid Family. An excellent example of gene duplication is found in the three branches of the opioid family. The large precursor molecule pro-opiomelanocortin (POMC) contains three similar amino acid sequences of α -, β -, and γ -MSH. POMC also contains ACTH and β -endorphin. Similarly, the proenkephalin molecule contains six copies of Met-enkephalin and one Leu-enkephalin sequence, while the third branch of the family, prodynorphin, contains three Leu-enkephalin sequences (Fig. 15.1). The ancestral gene for the opioids must have occurred very early in life as they are found in all living species, including invertebrates and plants. Opioid functions are discussed in Chapter 22 in Volume 2 of this handbook.

ACTH 1–39 and the peptide sequences within it (α -, β -, and γ -MSH) are collectively called the *melanocortins*. ACTH 1–39 possesses full biological activity, stimulating the adrenal cortex to produce glucocorticoids, which regulate carbohydrate and lipid metabolism, rapidly mobilizing carbohydrates from storage sites as well as suppressing the immune system. It also will darken the skin through the MSH

TABLE 15.2 Families of Mammalian Neuropeptides Based on Structural Similarities

Family	Abbreviation	Precursor
ACTH, MSH, and Opiates		
Adrenocorticotropin	ACTH	Pro-opiomelanocortin for all
Melanocyte-stimulating hormone	MSH	
β-Lipotropin		
β-Endorphin		
Bombesin-like Peptides		
Bombesin		Probombesin
Gastrin-releasing peptide	GRP	Pro-GRP
Neuromedin B	NMB	Pro-NMB
Rantensin		Pro-ranatensin RT
Calcitonin Gene-Related Peptides		
Calcitonin		Pro-calcitonin
Calcitonin gene-related peptide	CGRP	Pro-CGRP
Cholecystokinin		
Cholecystokinin	CCK	Pro-CCK
Gastrin		Progastrin
Enkephalins		
Met-enkephalin		Proenkephalin A
		Proenkephalin B
Leu-enkephalin		Proenkephalin A
		Proenkephalin B
Dynorphin		Proenkephalin B
Glucagon, Secretin		
Glucagon		Proglucagon
Secretin		Prosecretin
Vasoactive intestinal polypeptide	VIP	Pro-VIP/pro-PHI
Peptide histidine isoleucine	PHI	Pro-VIP/pro-PHI
Pituitary adenylate cyclase-activating peptide	PACAP	Pro-VIP/pro-PHI
Gastric-inhibitory peptide	GIP	Pro-GIP
Growth hormone-releasing hormone	GHRH	Pro-GHRH
Glycoprotein Hormones		
Thyroid-stimulating hormone	TSH	Pro-TSH
Follicle-stimulating hormone	FSH	Pro-LH/pro-FSH
Luteinizing hormone	LH	Pro-LH/pro-FSH
Chorionic gonadotropin	CG	Pro-CG
Insulin-like Growth Factors		
Insulin		
Insulin-like growth factors I and II		Proinsulin
Relaxin		Pro-IGF-I and -II
		Prorelaxin

(continued)

TABLE 15.2 (*Continued*)

Family	Abbreviation	Precursor
Neurotensin		
Neurotensin	NT	Proneurotensin/ proneuromedin
Neuromedin		Proneurotensin/ proneuromedin
Angiotensin II	AT-II	Proangiotensin
Oxytocin, Vasopressin		
Oxytocin	OT	Pro-OT/proneurophysin I
Vasopressin (antidiuretic hormone)	VP (ADH)	Pro-VP/proneurophysin II
Vasotocin		Provasotocin
Pancreatic Polypeptides		
Pancreatic polypeptide	PP	Pro-PP
Neuropeptide Y	NPY	Pro-NPY
Peptide YY	PYY	Pro-PYY
Somatotropins		
Growth hormone	GH	Pro-GH
Prolactin	PRL	Pro-PRL
Placental lactogen (choriomammotropin)	PL	Pro-PL
Tachykinins		
Substance P	SP	α -, β -, or γ -Protachykinins for these tachykinins
Neurokinin A	NKA	
Neurokinin B	NKB	
Tyr-MIF-1		
MIF-1		
Tyr-MIF-1		
Tyr-W-MIF-1		
Tyr-K-MIF-1		

Source: From [9], with permission from The MIT Press.

sequence (ACTH 1-13) and has potent neurotrophic influence on both the central and peripheral nervous systems. Separation of the peptide actions from the adrenocorticotrophic actions of ACTH 1-39 has been made possible by the separation or synthesis of noncorticotrophic fragments of the parent molecule, that is, chiefly α -MSH, and ACTH 4-9 and 4-10. These molecules are potent neurotrophic agents. They affect embryonic development and nerve regeneration, learning, memory, and attention in adults [5–7, 68–70]. An interesting bundling of different functions within a precursor molecule!

15.11.2.2 Gene Duplication in the Neurohypophyseal Family. The neurohypophyseal family of peptides OT and VP are found in all classes of vertebrates and all are nonopeptides. The VP-like peptides have in common a basic amino acid residue at position 8, whereas the OT-related peptides have a neutral amino acid in this

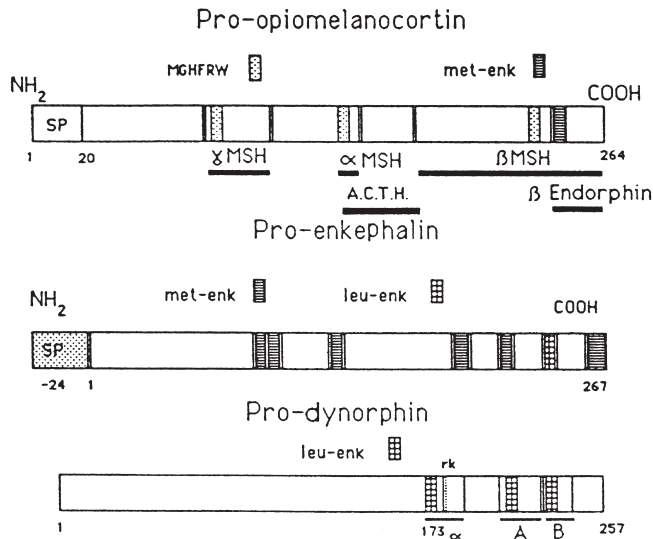


Figure 15.1 Structural relationships among prohormone, precursor forms of three main branches of opiate peptides, depicted as bar diagrams. The length in amino acid residues is indicated by the number at the corresponding C-terminus. Basic amino acids that are cleavage sites for processing are indicated by single or double vertical lines within the bars. SP, substance P; MSH, melanocyte-stimulating hormone; ACTH, adrenocorticotrophic hormone. (From [67], with permission from Oxford University Press.)

position. Duplication of the gene followed by mutation of residue 8 in one of the genes resulted in dual evolution of the two lines of peptides and their separate receptors (Fig. 15.2). Mammalian VP is probably derived from vasotocin, whereas the OT precursor was probably the mesotocin of amphibians. Precursor genes for these hormones existed in the animal kingdom before the divergence of vertebrates and invertebrates, perhaps 700 million years ago, and the processing machinery probably also predates divergence [71].

OT and VP are secreted by neurons in the supraoptic and paraventricular nuclei of the hypothalamus and stored in the posterior pituitary gland (neurohypophysis), from which they are secreted into the circulation. There is also a wide distribution of the axons of these hypothalamic neurons throughout the brain to regions that affect behavior and the regulation of reproductive events. In all species OT-like peptides are involved in reproduction and in mammals with milk ejection, uterine contractility, parturition, and female maternal, sexual, and social behavior. OT has behavioral properties opposite to those of VP; that is, OT may be an amnesic neuropeptide affecting consolidation and retrieval of memory, whereas VP has important effects on cognition [73] and has been reported to improve memory in diabetes insipidus patients [74].

VP-like peptides have antidiuretic and pressor functions. VP regulates water intake and diuresis and has important effects on the cardiovascular system. A mutation in the VP gene, if homozygous, results in diabetes insipidus, characterized by polyurea and polydipsia. Consequently *VP* is also referred to as antidiuretic hormone (*ADH*).

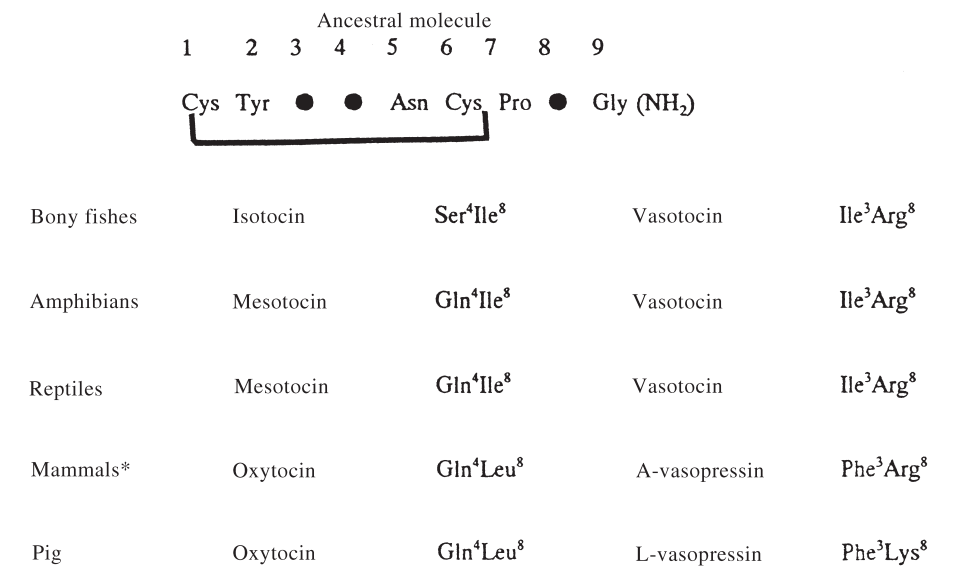


Figure 15.2 Hypothetical scheme of evolution of neurohypophyseal hormones. One gene duplication and a series of subsequent single substitutions in positions 3, 4, or 8 produce two molecular lines. The substituted amino acids and their positions in a hormone are listed to the right of each hormone. All oxytocin-like peptides have isoleucine, Ile in the 3rd position (Ile³), except pig. A, arginine; L, lysine. (From [72], with permission from The Royal Society (London).)

15.11.2.3 Duplication and Divergence in the NPY Family. NPY is a member of the pancreatic polypeptide (PP) family, which includes the gut endocrine peptide (PYY) and PP. NPY is found in abundance in the central and peripheral nervous systems. Each of these peptides consists of 36 amino acids and a C-terminal amide. NPY has been extremely well conserved during vertebrate evolution and is found in cartilaginous fish and even in the brain of cyclosomomes (lampreys), which diverged from the main vertebrate line about 450 million years ago [75]. The NPY family of homologous peptides arose as a series of gene duplications [76]. In contrast to the single ancestral NPY family gene, there are three Y subfamily receptors. Interestingly, these have evolved from seven Y receptor genes that resulted from chromosome quadruplication and subsequent loss of gene copies [77, 78]. NPY is involved in the regulation of many homeostatic functions as NPY neurons abundantly innervate the hypothalamus [79]. NPY is the most powerful physiological appetizer transducer known, and disorders in the complex humoral and circadian signals between NPY, leptin, and ghrelin promote hyperphagia and obesity [80]. The ability of PYY to curb appetite and its potential as an antiobesity drug have been topics of some controversy since first reported. McGowan and Bloom [81] conclude that while PYY has disadvantages in that it must be administered parenterally and has a short half-life, it has promising possibilities as an antiobesity drug in humans. NPY influences blood pressure, circadian rhythms, and sexual behavior. NPY also has been shown to modulate ethanol consumption [82]. There is considerable evidence that NPY acts to antagonize the behavioral consequences of stress [83], a topic that is discussed later in this chapter.

15.11.2.4 Gene Splicing. In addition to gene duplication, diversity of products in a single cell may result from splicing, a process in which introns, or parts of exons, are cut out. For example, the preprotachykinin gene can give rise to two mRNA molecules that code for SP and another two that code for both SP and another tachykinin, neurokinin (NK) [84].

15.12 ORGANIZATION OF NEUROPEPTIDE GENES

The isolation and characterization of POMC mRNA and the organization of the POMC gene have been extensively studied and serve as a model for most neuropeptides. The basic structure of the POMC gene has been highly conserved: It contains three exons and two introns. The large 3' exon contains the nucleotides coding for all the active neuropeptides. Exon 1 is a noncoding exon and exon 2 codes for the signal peptide [85]. The POMC gene has been localized on chromosome 2 in the human and chromosome 19 in the mouse [86]. Mutations affecting gene regulation may initiate or exacerbate neurological disease. New methods can rapidly and accurately identify likely neuropeptide gene regulatory regions using computer analysis of mouse, rat, and human genomic sequences. These identified sequences can then be analyzed in vivo using transgenic analysis. MacKenzie and Quinn [61] suggest that these technologies, combined with human polymorphic linkage analysis and association studies, may indicate how inappropriate regulation of neuropeptide gene expression may lead to human disease.

15.12.1 Regulation of Gene Expression

Neuropeptide gene expression is regulated by a complex series of negative and positive controls. In the anterior pituitary lobe, the secretion of POMC is stimulated by the hypothalamic secretion of CRH and VP and is inhibited by glucocorticoids from the adrenal cortex and from ACTH secreted by the anterior pituitary. Glucocorticoids decrease CRH mRNA in the paraventricular nucleus of the hypothalamus. In the intermediate lobe of the pituitary gland, the release of POMC is inhibited by the neurotransmitters dopamine and GABA. These secretory changes are preceded by appropriate increase or suppression of POMC mRNA activity in the pituitary. Hypothalamic POMC mRNA levels are also controlled by gonadal steroids, especially estrogen, which markedly reduces POMC mRNA [87].

15.13 BIOSYNTHESIS AND PROCESSING OF NEUROPEPTIDES

Like all polypeptides and proteins, neuropeptides are produced by cleavage from large precursor molecules synthesized on ribosomes and subsequently subjected to enzymatic proteolysis to yield neuropeptides of varying characteristics and potencies. Each peptide has an amino acid sequence determined by its corresponding gene. The nascent precursors are translocated through the intracellular membranes of the cell, chemically processed, packaged, and prepared for secretion.

15.13.1 Preprohormones

These are the initial precursor polypeptide chains that contain one or more amino acid sequences of the neuropeptide, one or more spacer (flanking) parts, plus the signal sequence at its N-terminus that guides it through the ribosome and into the lumen of the rough endoplasmic reticulum (RER). At this time the signal peptide is cleaved from the peptide chain by an endopeptidase [88]. The spacer parts lengthen the peptide to permit it to reach through the membranes to the inside of the RER.

15.13.2 Prohormones and Precursor Processing

The peptide chain with the signal peptide removed is the biologically inert *prohormone* containing one or multiple copies of the final active neuropeptides; thus this chain must be proteolytically processed into smaller active peptides which are then folded in the cisterna of the endoplasmic reticulum (ER) and shuttled to the *trans*-Golgi apparatus. Here they are packaged into secretory granules or vesicles that are budded off and in which posttranslational precursor processing occurs. This is the excision of peptide sequences from the prohormone by endopeptidases copackaged in the granules as the granules move down from the cell body to the nerve terminals [89]. The active peptide sequences to be excised are usually, but not always, flanked by dibasic amino acids, where cleavage occurs, first by endopeptidases, followed by exoproteolysis by amino- and carboxypeptidases, and in some cases by special amidating enzymes [90]. There may be additional enzymatic modifications such as glycosylation, phosphorylation, sulfation, or hydroxylation [91]. The precursor-processing endopeptidases are the *prohormone convertases* (PCs), which are found in all mammalian cells. Convertases are protease enzymes that clip off the active segments of the prohormone at single, specific basic residues or pairs of basic residues. There are several PCs, each of which is substrate specific [92, 93]. The prohormone convertase PC2 is believed to selectively convert multiple pro-neuropeptides into active peptides that function as neurotransmitters. Loss of PC2 results in changes of specific neuropeptides in certain tissues but not of all [94]. The specificity of the PCs and other proteases and their different distribution in neuroendocrine cells determine the time at which biologically active peptides are derived from the inactive precursor and thereby affect cellular development and activity [95]. Loss of the PCs in gene knockout experiments is lethal to embryos.

15.13.2.1 Role of Prohormones. Prohormones may have many different functions. Proinsulin ensures the correct folding and disulfide formation between the A and B chains of the insulin molecule. The neurophysins may serve a similar folding role for OT and VP. Prohormones vary considerably in size, but the size of the prohormone has little final relationship to the final size of the neuropeptide. The large prohormone proenkephalin gives rise to the five amino acid enkephalins. The complex prohormone POMC contains multiple neuropeptides that become active once the prohormone has been processed as well as some apparently biologically inert fragments (Fig. 15.1).

15.13.3 Tissue-Specific Processing

POMC was the first prohormone to be shown to be differentially processed in a tissue-specific way. In the anterior pituitary gland, cleavage of POMC by a PC yields chiefly a large N-terminal fragment ACTH 1-39 and β -lipotropin. In the intermediate lobe a different PC further hydrolyzes them to the shorter neuropeptides γ -lipotropin, β -endorphin, and α -MSH [92]. Thus the differential processing of a prohormone and its consequent yield may depend on variations in the relative amounts of the individual convertases with each tissue. Similarly, tissue-specific processing is found in the different cellular distribution of the PCs in the paraventricular nucleus and supraoptic nucleus, in which PC1 is colocalized with both OT and VP in intracellular secretory granules but PC5 is colocalized only with PC5. Differential processing of prohormones is an efficient method to achieve diversity in the nervous system.

15.14 INACTIVATION OF NEUROPEPTIDES

15.14.1 Intracellular Degradation

Degradation of neuropeptides to their inactive fragments or metabolites occurs in the RER or in the Golgi complex within about 30 min of their precursor synthesis, whereas later cleavages occur in the secretory granules in which the products may be stored for days. Thus the necessary enzymes must be present in the RER, Golgi complex, and secretory granules.

15.14.2 Extracellular Inactivation

Extracellular inactivation occurs rapidly through enzymatic degradation in plasma and by other cells, including glia, due to the widespread presence of peptidases. Most cell surface peptidases are anchored into the plasma membrane as integral membrane proteins. Those peptidases acting on small peptides belong to all groups of enzymes (endopeptidases, exopeptidases, and specialized peptidases), whereas those acting on larger proteins are endopeptidases that act internally in a peptide chain. A review of the structure and function of cell surface peptidases clarifies these differences [96].

An important cell surface peptidase, dipeptidyl-peptidase IV (DPP IV/CD26), selectively removes the N-terminal dipeptide with proline or alanine in the second position. Many neuropeptides are inactivated by DPP, making it an important physiological regulator, and DPP inhibitors, or peptide analogs resistant to DPP inactivation, are potential candidates for clinical use. The glucagon-like peptides (GLP-1 and GIP) are gastrointestinal peptides released after meals and are the most important secretins for insulin release. DPP rapidly degrades both peptides at the surface of endothelial cells or in plasma, resulting in a rapid loss of hormonal activity. DPP-resistant analogs of these neuropeptides theoretically could be of benefit in diabetic patients [97, 98].

Some cell surface proteases have dual effects: *inactivation* of one neuropeptide and *activation* of another. Angiotensin-activating enzyme (ACE) activates angiotensin I and inactivates bradykinin [99]. ACE activates angiotensin I, a decapeptide, by releasing a C-terminal dipeptide to form the octopeptide angiotensin II, a potent

vasopressor. Inhibitors of ACE are extensively used as antihypertensive drugs and for congestive heart failure. A similar role has been postulated for another cell surface endopeptidase, NEP, which may form functional complexes with ACE and peptide receptors on the cell surface that affect cellular signaling [100].

Neuropeptides are quickly inactivated in the gastrointestinal tract and are therefore ineffective if administered orally. However, peripherally administered neuropeptides bind tenaciously to cell membranes, which apparently protects them from proteolysis and permits them to perpetuate the cascade of receptor-mediated second-messenger effects for several hours.

15.15 NEUROPEPTIDE RECEPTORS

Neuropeptides, due to their insolubility in the cell membrane, must exert their effects by interacting with receptors on the cell surface. This interaction usually results in a change in the three-dimensional structure of the protein receptor, triggering a cascade of biochemical events in the cell and eventually leading to the specific cell response. Hormones, including neurohormones, exert their effects by binding to selective receptors on the target cell membrane. Hormone concentrations in vivo are normally very low, requiring that the receptor have a high affinity for that ligand. Hormone–receptor binding is reversible, resulting in the decay of the evoked effect after hormone removal or inactivation. A *high specificity* ensures that closely related neuropeptides, such as OT and VP, bind preferentially to their own receptors, but relative specificity is determined by the concentration of the hormone and its affinity of its receptor: At low concentrations VP will bind to its receptor and evoke pressor and antidiuretic effects; at high concentrations it can evoke the classic OT effects of uterine contractions and milk ejection.

Hormone receptors have the same properties as other cell surface receptors: They are finite in number so they are saturable, a characteristic of specific binding. However, receptor number can be increased (*upregulation*) or decreased (*down-regulation*) depending on the concentration of the hormone to which the receptor is exposed. Neuropeptides regulate the number and sensitivity of their own receptors. Insulin lowers the number of insulin receptors by decreasing receptor half-life [101].

15.15.1 Receptor Downregulation and Desensitization

Neuropeptides regulate the number and sensitivity of their own response. Desensitization is usually a decreased response to continued stimulation. Exposure to the agonist results in a peak in the response followed by a decline; subsequent exposure to the agonist evokes a smaller response, indicating desensitization due to sequestration of receptors (decreased receptor numbers) or loss of coupling to G proteins. After an extended time recovery occurs. Decreased receptor activity often involves phosphorylation of the receptor by a protein kinase, altering its conformation and changing its affinity for its ligand. Receptor phosphorylation is reversible by phosphatases.

Loss of receptors may also occur through endocytosis of the hormone–receptor complex after binding. This ends the first messenger signal and is an important part of desensitization. After acidification, the hormone is dissociated from its receptor

within the cytoplasm and receptors are returned to the Golgi apparatus, where they are recycled or degraded. Receptor number is also affected by the relative processes of receptor synthesis and degradation, actions that are affected by neuropeptides. Receptors are subjected to competition by closely related compounds and are profoundly affected by specific antagonists and drugs.

15.15.2 Receptor Upregulation and Sensitization

Chronic exposure to antagonists can increase receptor number. Several drugs such as lithium and antidepressant drugs alter the sensitivity of pre- and postsynaptic receptors, probably through an increase in neurotransmitter release.

15.15.3 Isoreceptors and Receptor Subtypes

Isoreceptors are structurally and functionally distinct receptors for the same hormone and all bind to the same natural hormone. They are mainly classified into subtypes by specific drug agonists and antagonists. The concept of multiple forms of receptors explains the subtle differences in the effects of closely related neuropeptides.

15.15.3.1 Arginine Vasopressin Receptors. The arginine vasopressin (AVP) receptor system is an example of neuropeptide receptor subtypes. AVP V1 and V2 receptors are differentially distributed in tissues and use different second messengers. The V1 receptor is widely distributed in brain and peripheral tissues and elicits glycogenolysis in liver and vasoconstriction in vascular smooth muscle through a cAMP-independent mechanism coupled to phosphoinositol turnover [102]. An effect on cardiac cells is also indicated as VP directly induces myocyte growth through the V1 receptor in neonatal rat heart cells [103]. The V2 receptor is coupled to cAMP and is located in the kidney where it mediates the important antidiuretic effects of AVP [104].

15.15.3.2 Neuropeptide Y Receptor System. In mammals this consists of three peptides and four to five G-protein-coupled receptors (GPCRs). The molecular evolution of the NPY receptor subtypes from seven Y receptor genes in gnathosomes to four to five in mammals is discussed by Larhammar and Salaneck [77]. NPY may act on virtually all hypothalamic neurons via one or several receptors, explaining the ability of NPY to regulate hypothalamic–pituitary axes influencing homeostatic behavioral and physiological functions, including appetite regulation, circadian rhythm and anxiety, as well as involvement in the response to stress. These receptors have been cloned and their differential distribution in the hypothalamus determined by various histological techniques [79, 105].

15.15.3.3 Tachykinin Receptors. The receptors of SP, neurokinin A, and neurokinin B have been cloned and classified into three groups: NK₁ (the predominant receptor for SP), NK₂, and NK₃, all of which are GPCRs. GPCRs may exist in a wide variety of different conformations, each of which has a different ability to recognize, desensitize, and stimulate effector systems, thus enabling the receptor to activate one or more G proteins in a ligand-specific manner [106–108]. Tachykinin are involved in a multitude of physiological functions due to their widespread

distribution, both centrally and peripherally. Peripherally tachykinins regulate blood flow and vascular permeability, salivation, and micturition. They are potent constrictors in smooth muscle and consequently are involved in gastrointestinal motility and intestinal secretions and are potent spasmogens of airway smooth muscle. Through the action of SP they function as pain transmitters from the periphery. Centrally, tachykinins act as neurotransmitters and neuromodulators in the spinal cord and SP regulates processes involving sensory perception (vision, olfaction, and audition) in addition to pain.

15.15.3.4 Melanocortin Receptors. Five types of melanocortin receptors have been cloned, and the markedly different effects of the melanocortins and various synthetic analogs on the adrenal cortex, melanoma cells, CNS neurons, and peripheral neurons can be explained on the basis of the differential distribution of the receptors in these tissues. See Adan and Gispen [109] and Strand [7, p. 283] for overviews. MC-1R is the MSH receptor and is specifically expressed in melanocytes and melanoma cells. MC-2R is an ACTH receptor and is localized in the adrenal cortex but also activates all MCRs. MC-3R, MC-4R, and MC-5R are the three neural receptors and their distribution may account for their effect on neural development, regeneration, and neuroprotection [109–111]; [7, p. 285]. The important role of brain α -MSH receptors and leptin in regulating food intake is discussed in Chapter 25 in Volume 3 of this handbook. Leptin regulates several neuropeptide genes, including POMC and NPY, and stimulates the hypothalamic expression of α -MSH, an orexigenic peptide, while inhibiting the release of melanocyte-concentrating hormone, an anorexic peptide [112].

15.16 NEUROPEPTIDE RECEPTORS AND SECOND-MESSENGER SYSTEMS

There are several different classes of neuropeptide receptors, classified on the basis of their structure and the second-messenger systems that they activate.

15.16.1 G-Protein-Coupled Receptors

More than 80% of neuropeptide receptors, most of which are glycosylated, are coupled to G proteins and stimulate adenylate cyclase and cAMP formation. GPCRs are characterized by a core of seven membrane-spanning helices connected by alternating cytoplasmic and extracytoplasmic loops with a central pore exposed to the extracellular surface. The specificity of the receptor depends on its distinct extracellular ligand binding domain. GPCRs are being intensely studied as potential targets of drug research due to the size and diversity of this receptor family as well as the high levels of specificity and sensitivity of the drugs affecting these receptors. GPCRs have recently been reviewed [113].

15.16.2 Phospholipase–Phosphatidylinositol Linked Messengers

The hydrolysis of PIP_2 is stimulated by the neuropeptides TSH, bombesin, VP, and GnRH, resulting in a cascade of phosphorylations that ultimately result in the specific response of the biochemical machinery of the cell.

15.16.3 Guanylate Cyclase (cGMP) Receptors

cGMP, unlike cAMP, is found in both the membrane and the cytoplasm. The soluble form of the enzyme is activated by nitric oxide (NO) and free radicals. Membrane-bound cGMP forms a transmembrane receptor complex for atrial natriuretic hormone (ANH), an important neuropeptide involved in electrolyte balance and cardiovascular homeostasis. Three natriuretic receptor subtypes have been cloned [17]. The ANH receptor has only one membrane-spanning helix and the extracellular binding site for neuropeptides is directly linked to the catalytic cGMP domain. cGMP is limited in its distribution with its highest concentration in the cerebellum, atrium of the heart, and kidney.

15.16.4 Tyrosine Kinase–Coupled Receptors

These receptors have only one membrane-spanning helix. The binding site is in the extracellular domain and the tyrosine kinases act by initiating a cascade of protein phosphorylations that are particularly important during development and growth. These are the receptors for insulin, insulin-like growth factor, and several other growth factors [114].

15.16.5 Cytokine Receptors

Cytokine receptors are transmembrane glycoproteins which are classified into various families. Type I consists of growth hormone (GH) and prolactin (PRL) and some of the interleukins, erythropoietin and ciliary neurotrophic factor. These receptors share some similarities with the tyrosine kinase receptors in that they have only one transmembrane helix.

15.17 HYPOTHALAMIC CONTROL OF THE PITUITARY GLAND

15.17.1 Hypothalamic Release-Stimulating and Release-Inhibiting Neuropeptides

Neuropeptides are especially concentrated in the CNS and the hypothalamus, median eminence, and pituitary gland contain the highest concentration of neuropeptides.

Hypothalamic control of anterior pituitary secretions is through neuropeptides that stimulate the release of pituitary hormones (RH) and those that inhibit pituitary secretion (IH). The *release-stimulating hormones* include CRH, GnRH, TRH (thyrotropin-releasing hormone), and growth hormone-releasing hormone (GHRH). The best evidence for a *release-inhibiting factor* is SRIF (somatotropin release-inhibiting factor), also called somatostatin, which inhibits the release of growth hormone, or somatotropin.

15.17.1.1 Control of Prolactin Release. Hypothalamic dopamine is the most potent *inhibitor* of prolactin release. However, a 38-residue hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide (PACAP) is a potent *stimulator* of PRL release through its action on adenylate cyclase in the pituitary. PACAP stimulates PRL gene expression and POMC gene transcription. PACAP also stimulates

gonadotropin release from pituitary cells [115], and due to its widespread distribution in many tissues, PACAP has been shown to have important effects in malignant diseases and in immunology and inflammation [116, 117].

15.17.2 Hypothalamic–Pituitary Pathways

Hypothalamic control over the pituitary gland uses two different routes. In one route neurons from the dorsomedial and ventromedial nuclei (the parvocellular neurosecretory system of the hypothalamus) deposit their secretions in the medial eminence, where they enter a special *portal system* that leads to the anterior pituitary. Thus hypothalamic releasing and release-inhibiting neuropeptides pass through this special circulation, for example, GnRH, GHRH, CRH, TRH, and somatostatin. This is the *parvocellular system*. The second system, the *magnocellular system* [7, p 78], delivers the hypothalamic hormones OT and VP directly into the posterior pituitary through axons from cells within the paraventricular and supraoptic nuclei that extend through the infundibulum to the posterior pituitary.

15.17.3 Additional Pathways for Neuropeptide Delivery

Other pathways exist that indicate the complexity of the delivery of neuropeptides. The portal system may shunt blood retrogradely to the anterior pituitary, hypothalamus, and other brain regions, permitting hypothalamic and pituitary neuropeptides to reach distant parts of the brain through cerebrospinal fluid (CSF) flowing through the ventricles and the subarachnoid spaces. Specialized cells (tanycytes) in the floor of the third ventricle control access from neuropeptide-containing nerve terminals to the portal blood vessels. These various systems provide a selection of routes by which neuropeptides reach the brain and may affect phenomena as diverse as sleep, pain, orgasm, and headache [118].

15.18 BLOOD–BRAIN BARRIER

The BBB forms an effective structural barrier to the passage of large neuropeptides yet there is convincing evidence that systemically administered neuropeptides have potent effects on the CNS [1, 120, 121]. There are three main mechanisms by which neuropeptides cross the BBB and exert central effects:

1. *Penetration via Pores and Pinocytosis* The tight junctions in the BBB are not completely leakproof as a small amount of albumin can be seen in the CSF but it is only about 0.5% of plasma albumin concentration.
2. *Transmembrane Diffusion* Diffusion through the endothelial membrane depends on many physicochemical properties, including lipid solubility, molecular weight, ionization, and the ability to form electroneutral complexes. Several neuropeptides cross the BBB by this route, for example, the tripeptide TSH, MSH, insulin, and delta sleep-inducing peptide [122–125].
3. *Specific Carrier-Mediated Mechanisms* Water-soluble molecules such as glucose are transported almost exclusively by specific carrier-mediated transport

systems. These systems are saturable and highly stereospecific. Most transport systems are found in the choroid plexus or the capillary bed of the CNS. The specificity of these transport systems is indicated by the more than 10-fold variation in penetration for gastrin, delta sleep-inducing peptide, VIP, and calcitonin. Some neuropeptides are transported out of the brain but most are transported into the brain [123].

The peptide transport system-1 (PTS-1) transports small peptides with an N-terminal tyrosine, such as Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH₂), the analgesic peptides Met- and Leu-enkephalin, and dynorphin 1-8. A second system transports VP-like peptides in both directions. Arginine VP crosses intact from blood to brain and from brain to blood and also regulates the entry of several amino acids. Bradykin, angiotensin, and cytokines increase the permeability of the BBB to other neuropeptides. As a result of the differential penetration of neuropeptides, the composition of CSF is very different from that of plasma. The important role of the choroid plexuses in peptide transport and their significance in the delivery of drugs and peptide analogs to the brain are discussed by Smith et al. [126].

Neuropeptide penetration of the BBB may be increased by altering the physico-chemical properties of the synthetic neuropeptides, and their biological activity may be prolonged by peptidase inhibitors. The BBB may be modified by neurotoxins, neurotropic viruses, age, stress, and diurnal rhythms. It has been suggested that a faulty BBB may play a role in Alzheimer's disease, epilepsy, and paranoid psychosis.

15.19 ADMINISTRATION OF NEUROPEPTIDES

Oral administration of neuropeptides is ineffective due to inactivation by gastric and plasma peptidases. Peripheral administration by intraperitoneal injection is effective [3, 68, 127, 128], but due to the low penetration of the neuropeptides through the BBB, the concentration that reaches the brain may be insufficient in some cases. Subcutaneous injection is not effective. The method of choice now is central administration through intracerebroventricular or intrathecal injection. For chronic central administration, mini-osmopumps attached to intrathecal cannulae are used [129, 130]. Intranasal injection of some neuropeptides (e.g., VP) is highly efficient and the obvious choice of delivery clinically [131]. The disadvantage of all these methods is that they deliver either a single dose or a continuous dosage, whereas the physiological method is through pulsatile release of most hormones. Also the inverted *U-shaped dose-response curve* for administered neuropeptides is an important factor to remember when increasing the concentration of the peptide—higher dosages may decrease efficiency or even be ineffective [132].

15.20 TIME- AND TISSUE-SENSITIVE RESPONSES TO NEUROPEPTIDES

The effectiveness of neuropeptides may vary with the metabolic milieu, stage of development of the target tissue, and critical interaction with other growth factors, including the sex steroids. In both growing and regenerating systems, the melanocortins, for example, enhance growth processes but nevertheless constrain these

dynamic systems within normal physiological limits. They have little if any effect on stable, healthy full-grown neuromuscular systems. Hypophysectomized animals may respond robustly to the administration of neuropeptides that are ineffective in normal, healthy controls [132]. It is important to realize that neuropeptides may exert their main actions when the nervous system is stressed, challenged, or afflicted by disease [133–135].

15.21 NEUROPEPTIDE REDUNDANCY

There appears to be much redundancy in the actions of neuropeptides [136]. For example, many different neuropeptides (e.g., NPY, melanocortins, galanin, TSH, NPY, among others) have been reported to be active in nerve regeneration and development. The extensive evidence that melanocortins accelerate recovery from nerve trauma and affect the development of the neuromuscular system is described in several reviews [6, 69, 70]. However, these remarkable effects have not been translated into effective clinical trials. As similar neuroregenerative and neuroprotective effects have been described for several other neuropeptides, it is difficult to dissect out which particular neuropeptide is the most important. The failure of some knockout animals to display defects in phenotypes is particularly disturbing to the concept of neuropeptide-specific activity: One can only assume that a “fail-safe” redundancy has evolved to replace missing neuropeptides.

15.22 STRESS AND NEUROENDOCRINE RESPONSE

The hypothalamic–pituitary–adrenal (HPA) axis is the coordinator of the stress response, translating neural, endocrine, and cytokine information into physiological responses. Several CNS sites (e.g., cerebral cortex, amygdala, and hippocampus) deliver neural input to the hypothalamus [137]. In the hypothalamus these stimuli are transduced to neuroendocrine responses through the sequential stimulation of CRH, which together with the synergistic release of AVP and OT causes the secretion of ACTH, MSH, and the endorphins by the anterior pituitary. Endorphins, pain suppressors, are released in equimolar quantities as ACTH as a result of their simultaneous processing from POMC. Teleologically, this is a useful mechanism for ignoring pain while responding to stress in a “fight-or-flight” manner first described by Cannon and de la Paz [138]. These authors emphasized the role of the sympathetic nervous system in response to stress and it is this system, through the release of catecholamines, which is intimately involved in the activation of hypothalamic release of CRH. In turn, CRH activates the sympathoadrenal system as well as causing the release of pituitary ACTH, MSH, and the endorphins [139]. Catecholamines and the glucocorticoids together inhibit the immune system [140]. Either an over or understimulation of the HPA axis can have drastic effects. A hyperresponsive stress system, with prolonged exposure to ACTH-evoked glucocorticoids as a result of chronic stress, can cause hypertension and immunosuppression, with consequences seen only in a stressed or pathological condition. This may be due to the redundancy of some neuropeptides, leading to the assumption of the existence of a

“fail-safe” mechanism to vulnerability to inflammatory disease, hyperinsulinism and insulin resistance, hypertension, tachycardia, and Cushing’s disease. Glucocorticoids can have a wide range of deleterious effects on the brain, predominantly in the hippocampus [141]. A hyporesponsive stress system can be equally devastating, resulting in seasonal depression, chronic fatigue, and increased susceptibility to autoimmune and inflammatory diseases [142]. Catecholamine levels increase sharply in acute stress enabling the organism to tolerate the physiological trauma, but if the stress is prolonged or severe, catecholamine-caused vasoconstriction can be fatal [143]. Stress thus involves both the hypothalamic CRH circuits and the norepinephrine-containing brain neurons [139]. The entire stress-evoked circuits are replete with important negative- and positive-feedback systems.

An additional regulatory circuit evoked by stress involves the novel neuropeptide CART (cocaine- and amphetamine-regulated transcript) that may provide a link between the HPA, stress, and psychostimulant drugs. CART mRNA is increased in the striatum after the administration of cocaine and amphetamine, and CART is found in areas that regulate CRH, feeding and sensory processing, and other important physiological processes (for reviews see [144–148]). CART can affect CRH release from CRH-containing neurons in the paraventricular nucleus (PVN) and injection of CART into the PVN increases plasma ACTH and corticosterone [149]. CART also appears to be regulated by glucocorticoids [150]. The existence of these neuroendocrine links indicates the presence of multiple regulators of the organism’s ability to respond to stressful situations.

15.23 SUMMARY

The identification, characterization, and evolution of neuropeptides, neuropeptide families, and their receptors are discussed, including new information gleaned by genetic manipulations, peptidomics, and genomics. The flexibility of neuropeptides to act as neurohormones, neurotransmitters, and/or neuromodulators in regulating and modifying synaptic events is emphasized. Neuropeptide processing and inactivation are considered and the interesting phenomenon of tissue-specific processing of prohormones is introduced as an efficient method to achieve diversity in the nervous system. The hypothalamic control of the pituitary gland is outlined, as is the complexity of the pathways that permit the delivery of neuropeptides between these organs and to other sites in the brain. While the BBB prevents the passage of large neuropeptides, there are several mechanisms by which peripherally administered neuropeptides cross the BBB and exert central effects. Intracerebroventricular, intrathecal, and intranasal are other routes of effective administration of neuropeptides. Most studies have shown that the effectiveness of neuropeptides varies with the stage of development of the target tissue and the metabolic milieu, and many neuropeptides may be effective in replacing missing or defective neuropeptides. The vital role of the HPA axis in the coordination of the stress response and the clinical significance of a hyporesponsive versus a hyperresponsive HPA axis, involving the sympathoadrenal system, demonstrate the importance of the many negative- and positive-feedback systems that control these adaptive responses.

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16

NEUROTRANSMITTER TRANSPORTERS

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16.1	Neurotransmitter Transporter Families	707
16.1.1	Plasma Membrane Neurotransmitter Transporters	707
16.1.1.1	Plasma Membrane Glutamate Transporters (SLC1)	708
16.1.1.2	Na ⁺ / Cl ⁻ -Dependent Neurotransmitter Transporters (SLC6)	711
16.1.1.3	Choline Transporters (SLC5)	714
16.1.1.4	Glutamine Transporters (SLC38)	715
16.1.2	Vesicular Neurotransmitter Transporters	715
16.1.2.1	Vesicular Glutamate Transporters (SLC17)	716
16.1.2.2	Vesicular Acetylcholine and Monoamine Transporters (SLC18)	716
16.1.2.3	Vesicular Inhibitory Amino Acid Transporters (SLC32)	718
16.2	Clinical Relevance of Neurotransmitter Transporters	718
16.2.1	Plasma Membrane Glutamate Transporters	718
16.2.2	Plasma Membrane Monoamine Transporters	719
16.2.2.1	Serotonin Transporters	720
16.2.2.2	Dopamine Transporters	721
16.2.2.3	Norepinephrine Transporters	721
16.2.3	Plasma Membrane GABA Transporters	721
16.2.4	Plasma Membrane Glycine Transporters	722
16.2.5	Vesicular Transporters	723
16.2.6	Interactions Between Neurotransmitter Systems	723
16.3	Plasma Membrane Neurotransmitter Transporter Regulation	724
16.3.1	Chronic Regulation	724
16.3.1.1	Polymorphisms	724
16.3.1.2	Multiple Transcription Initiation Sites	725
16.3.1.3	Glycosylation	725
16.3.1.4	Chronic Substrate Treatment	726
16.3.2	Acute Regulation	727
16.3.2.1	Second Messengers	727
16.3.2.2	Agonists and Antagonists	728
16.3.2.3	Interacting Proteins	728
	Acknowledgments	729
	References	729

The now classic “*Vagusstoff*” studies of Otto Loewi in the 1920s provided the evidence for chemical-based signaling in the nervous system, and experiments performed in the decades that followed gave identities to many of these neurotransmitters. However, as recently as the 1950s, the fate of transmitter after its release from the presynaptic nerve terminal was still unclear. It was certainly known that transmitters such as acetylcholine (the *Vagusstoff*) were subject to enzymatic degradation. Less clear was whether neurotransmitter could also be recycled to the presynaptic neuron via some reuptake process. This possibility was verified by Hertting and Axelrod approximately four decades ago. They found that the synaptic actions of norepinephrine were attenuated by reuptake back into the nerve terminal from which it was released [1]. Subsequently, the reuptake process was found to be saturable and of high affinity, suggesting a specific process mediated by transporter proteins [2]. Thus the field of neurotransmitter transport was born. Elegant biochemical and pharmacological studies soon followed which delineated many of the functional properties of the transport process, and molecular biology approaches identified the different gene and protein families that act to move neurotransmitters across lipid membranes in brain.

We have learned much about the carrier-mediated transport of neurotransmitter in the past half-century. We know that some transporters reside on the plasma membrane of neurons and glia where they control ambient extracellular transmitter levels. Some plasma membrane transporters are expressed at high density at or near synapses where they can regulate neuronal signaling by sequestering transmitter away from neurotransmitter receptors and by preventing transmitter from spilling over to neighboring synapses. We know that these transporters recycle transmitter for rerelease or provide transmitter precursors for the synthesis of new transmitter. For example, presynaptic choline transporters provide choline for the synthesis of acetylcholine. Two glutamine transporters, one to efflux glutamine from glia and one to take up glutamine into neurons, are part of the glutamate–glutamine shuttle and are necessary for the synthesis of the amino acid transmitters glutamate and γ -aminobutyric acid (GABA). But neurotransmitter transporters are not only found on cell membranes; we know that transporters are also found on the membranes of transmitter-containing synaptic vesicles, where they act to fill synaptic vesicles from pools of cytoplasmic transmitter for subsequent activity- and calcium-dependent release.

We know that neurotransmitter transporters must typically move transmitter against the transmitter concentration gradient, and to do this they couple transmitter uptake to the electrochemical gradient of associated ions. We know that some transporters not only act as carriers of transmitter but also are permeable to ions, with conductances akin to those of ion channels; these different modes of activity suggest that transporters may have functions in neurons and glia that are distinct from their carrier roles. We know that transporter function can be regulated, that the regulation can affect the trafficking and subcellular distribution of transporters as well as their ability to flux transmitter, that the regulation can be both acute and chronic, and that this regulation can have physiological consequences.

We also know that these transporters represent the targets of drugs of abuse such as cocaine, amphetamine, and Ecstasy and therapeutic drugs for the treatment of diseases such as depression and epilepsy. We know that transporters can operate in reverse under certain conditions, which can be important in both normal and

abnormal neuronal function. We know that transporters are crucial for normal physiological activity in animals because genetically manipulated animals that lack particular transporters have striking phenotypes. And we know that naturally occurring transporter mutations and abnormal transporter function are associated with a variety of clinical phenotypes in humans.

In this chapter, we review many of these issues, beginning with a general examination of the different families of neurotransmitter transporters. We then examine the associations between transporter function, disease states, and clinical phenotypes. Finally, we provide two examples of chronic and acute regulation of transporter function.

16.1 NEUROTRANSMITTER TRANSPORTER FAMILIES

As discussed above, neurotransmitter transporters function to permit the movement of transmitter across biological membranes in both the peripheral and central nervous system (CNS). One can separate transporter families by where functional transporters are expressed. Plasma membrane neurotransmitter transporters of neurons and glia carry transmitter between the extracellular space and the cell cytoplasm; vesicular transporters in neurons carry transmitter between the cytoplasm and the vesicle interior.

Because neurotransmitter transporters allow for the electrochemical-driven movement of solutes across membranes, they have been assigned, based upon the Human Genome Nomenclature Committee Database, to the solute carrier (SLC) gene family, which includes 43 subfamilies and 298 transporter genes. A transporter is assigned to a specific SLC family if it has at least 20–25% amino acid sequence identity to other members of that family [3]. We subdivide our review of transporters based upon this classification scheme.

16.1.1 Plasma Membrane Neurotransmitter Transporters

The fidelity of synaptic transmission at chemical synapses depends on many factors. Two of these factors include maintaining low levels of ambient extracellular transmitter in order to prevent receptor overactivation or desensitization and rapidly sequestering transmitter away from receptor sites following transmitter release. These functions are carried out in part by transporters that reside on the plasma membrane of pre- and (in some cases) postsynaptic neurons or perisynaptic glial elements. In mammals, each small-molecular-weight neurotransmitter (the excitatory amino acid glutamate, the inhibitory amino acids GABA and glycine, and the monoamines dopamine, serotonin, and norepinephrine) has its own highly specific transporter proteins to carry out this action. The SLC1 (Na^+ -dependent) transporter family includes five transporters for glutamate. The SLC6 (Na^+/Cl^- -dependent) transporter family consists of genes that encode four GABA transporters, two glycine transporters, and one for each monoamine. In addition, there are three plasma membrane transporters for transmitter precursors. These include the choline transporter and two glutamine transporters. The general cellular localization of transporters at GABAergic and glutamatergic synapses is depicted in Figure 16.1.

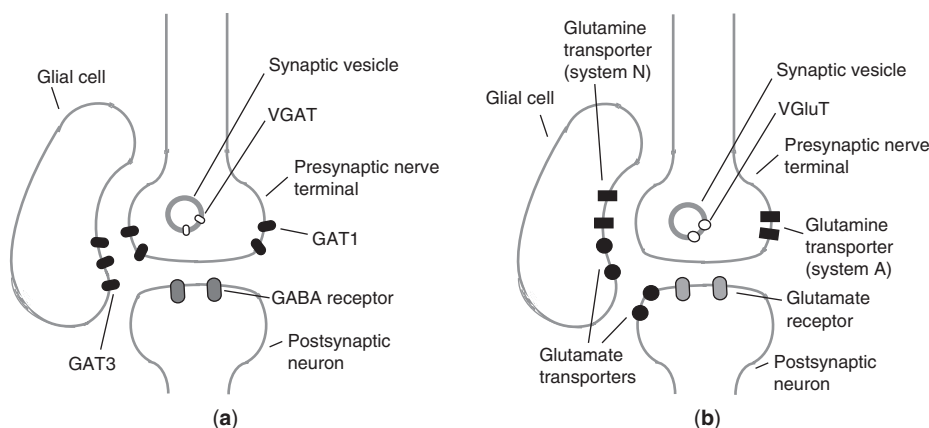


Figure 16.1 Localization of neurotransmitter transporters. (a). GABA transporters (GATs) are localized on presynaptic nerve terminals of GABA neurons as well as glial cells at these synapses. GAT1 is predominantly expressed perisynaptically on the nerve terminal, while GAT 3 is localized to glial cells. The vesicular GABA transporter (VGAT) is expressed on vesicles in the presynaptic terminal, where it is responsible for the packaging of GABA for exocytosis. (b) At glutamatergic synapses, glutamate transporters are located on glial cells, as well as pre- and postsynaptic neurons. Excitatory amino acid transporters EAAT1 and 2, the predominant glutamate transporters, are expressed primarily on the glial cells surrounding the synapse. Other members of the glutamate transporter family are expressed more specifically, such as EAAT4, which is localized perisynaptically on postsynaptic Purkinje cells of the cerebellum. Glutamine transporters, which are required for uptake of the glutamate precursor molecule, are located both on presynaptic neurons and glial cells. System A subfamily glutamine transporters are found primarily on neurons, while system N are localized to astrocytes. Vesicular glutamate transporters (VGLUTs) are located intracellularly on the plasma membranes of neurotransmitter-containing vesicles.

16.1.1.1 Plasma Membrane Glutamate Transporters (SLC1). The SLC1 family consists of five high-affinity excitatory amino acid transporters (EAATs) [4, 5] for glutamate and two neutral amino acid transporters, ASCT1 and 2. EAAT1 (also known as GLAST, glutamate–aspartate transporter) and EAAT2 (also known as GLT-1, glutamate transporter) are the predominant glutamate transporters in glia, with EAAT1 prevalent in cerebellum and EAAT2 in forebrain. In contrast, EAAT3 and 4 are primarily expressed in neurons, with EAAT3 widely expressed in various brain areas and EAAT4 mostly confined to the dendrites of Purkinje cells in the cerebellum [6, 7]. Thus, EAAT4 is a postsynaptically localized transporter. EAAT5 is present in both neurons and glia in the retina. It is associated with rod photoreceptors and bipolar cells and is therefore thought to participate in the processing of visual information [8]. Neuronal EAAT3 and EAAT4 are typically localized extrasynaptically and contribute less significantly to glutamate uptake in the brain than the two astroglial transporters. EAAT3 is also localized to presynaptic GABA-containing terminals and may play a role in providing glutamate for GABA metabolism [9].

The predominant substrates for glutamate transporters are L-glutamate and D/L-aspartate. During each transport cycle, three Na^+ ions and one H^+ ion are

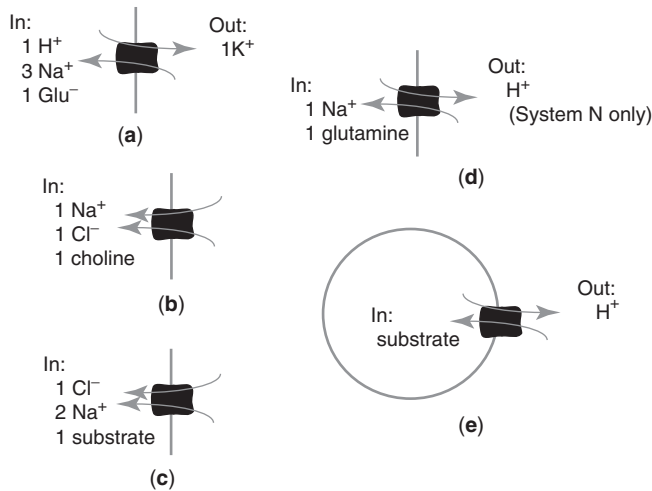


Figure 16.2 Stoichiometry of neurotransmitter transporters. (a) The SLC 1 family includes the five high-affinity glutamate transporters (EAAC1, GLT-1, GLAST, EAAT4, and EAAT5), which transport glutamate and aspartate into the cell accompanied by cotransport of 1 H^+ and 3 Na^+ and countertransport of 1 K^+ . (b) Choline transporters are members of the sodium/glucose transport (SLC5) family, which includes 220 members in the animal and bacteria kingdoms. The choline transporter (SLC5A7) couples the transport of choline to the influx of Na^+ and Cl^- down their gradients. (c) The SLC6 family contains the transporters for GABA, glycine, norepinephrine (NE), 5-hydroxytryptamine (5-HT), and dopamine (DA). These transporters generally couple substrate transport to the influx of Na^+ and Cl^- , although the serotonin transporter also transports a K^+ ion along with this cargo. (d) The SLC38 family contains two types of glutamine transporters, divided into system A and system N. System-A-type glutamine transporters likely cotransport their substrate along with 1 Na^+ , while system-N subfamily transporters likely cotransport a Na^+ ion and antiport a proton. (e) Vesicular neurotransmitter transporters are grouped into SLC families 17, 18, and 32. These transporters are driven by the electrochemical gradient that is generated by the vacuolar ATPase (V-ATPase), which translocates protons into the vesicle. The vesicular transporters use this gradient to transport substrate into the vesicle coupled to the outward transport of one or more H^+ ions.

cotransported along with the amino acid, and one K^+ ion is countertransported (Fig. 16.2a). This stoichiometry of transport permits a transmembrane glutamate concentration gradient ($[\text{Glu}]_{\text{in}}/[\text{Glu}]_{\text{out}}$) of approximately 100 to be achieved by glutamate transporters under normal circumstances; thus, glutamate transporters can maintain an ambient extracellular glutamate concentration in the low-nanomolar range [10]. When this electrochemical gradient collapses under pathological circumstances, such as during ischemia, the equilibrium state of the transporter shifts, and extracellular glutamate concentrations can rise to high-micromolar levels [11]. The reliance of transporters on the electrochemical gradient for transmitter transport underlies the hypothesis of transporter-mediated excitotoxicity, a leading theory for massive neuronal death observed in various pathological states [12–14]. This phenomenon will be further discussed in relation to its clinical implications later in this chapter.

Structurally, hydropathy analysis predicts a protein topology of eight transmembrane domains (TMDs) connected by alternating extracellular and intracellular

loops, intracellular N- and C-termini, and a large extracellular loop between TMDs 3 and 4 [15, 16]. A unique, highly conserved and long hydrophobic stretch of amino acids is located near the C-terminus and is believed to be responsible for substrate binding and translocation. Between TMDs 7 and 8, there is a “reentrant loop” that likely forms part of the pathway for the translocation of substrates and cotransported ions, similar to that found in many ligand- and voltage-gated ion channels [17] (Fig. 16.3a). The three-dimensional structure of glutamate transporters predicted by freeze-fracture electron microscopy suggests that functional EAAT3 is expressed as a distinct pentagonal 10-nm-diameter particle in the plasma membrane [18], supporting evidence for an oligomeric structure to glutamate transporters [19]. Recently, the crystal structure of a bacterial homologue to plasma membrane glutamate transporters was reported [20]. This transporter has a trimeric structure with a bowl that extends down to the midpoint of the lipid bilayer. At the base of the bowl are binding sites. The movement of nearby helices may allow for these binding sites to have alternating access to each side of the membrane.

How transporter structure maps onto transporter translocation of substrates has been of considerable interest not only for glutamate transporters but also for all neurotransmitter transporters. The classic translocation model is that of alternating access: Substrates attach to their binding sites in a stoichiometric fashion on one side of the membrane; a conformational change of the protein then occurs such that the binding sites are relocated to the opposite side of the membrane; lastly, the substrates unload and the transporter resets to its initial state. This model accounts for some but not all of the recent physiological data. To account for these data, more recent models argue for either variations in alternating access [21, 22] or transport schemes more similar to flux through ion channels [23]. Although the glutamate transport

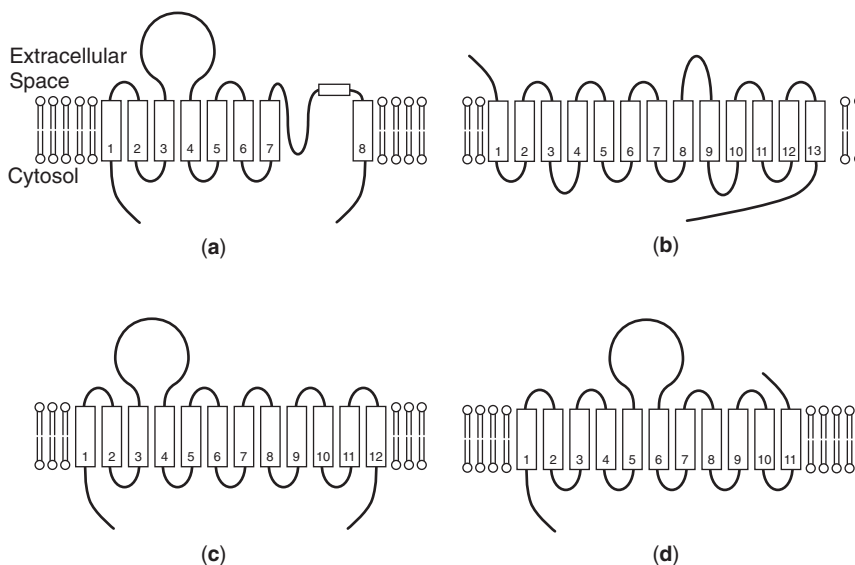


Figure 16.3 Schematic topology of plasma membrane transporters: (a) SLC 1 (glutamate transporters); (b) SLC 5 (choline transporters); (c) SLC 6 (GABA, glycine, and monoamine transporters); (d) SLC 38 (glutamine transporters).

cycle described above is consistent with the alternating access model, it is also known that glutamate transporters can behave as glutamate-activated Cl^- channels; Cl^- is not required for glutamate transport [24]. These glutamate-activated Cl^- currents regulate membrane potentials and affect synaptic signaling [25].

The indispensable role of glial glutamate transporters in controlling extracellular glutamate levels is prominently illustrated in GLT1 or GLAST knockout mice. Less than 10% of total glutamate transporter is retained in the CNS of GLT1 null mice. These mice suffer from lethal spontaneous seizures and increased susceptibility to acute cortical injury. Most of them die by postnatal week 6 [26]. Knockout of GLAST, a transporter that is abundant in cerebellum, leads to impairment in coordinated motion and increased cerebellar vulnerability after acute brain injury [27].

Once taken up by astrocytic glutamate transporters, glutamate is metabolized via two major pathways: conversion to glutamine or entrance into the tricarboxylic acid (TCA) cycle. Formation of glutamine, which is the precursor not only for glutamate but also for GABA and glycine, is catalyzed by glutamine synthetase. Alternatively, glutamate can function as a substrate for the TCA cycle, in which it is converted to 2-oxoglutarate (2-OG) for energy metabolism [28]. Recent evidence also connects glutamate transport with the classic role of astrocytes in providing lactate to neurons as an energy source. In this model [29], glutamate released upon synaptic activity is taken up by astrocytes. Three Na^+ ions cotransported with glutamate into the astrocyte trigger adenosine triphosphatase (ATPase) activity to extrude the intracellular sodium load. The drop in ATP levels leads to aerobic glycolysis in which lactate is synthesized. The lactate is then shuttled back to neurons to fuel neuronal activity, including synaptic transmission. Glucose utilization and lactate secretion are decreased in GLT1 and GLAST mutant mice. This reduction in glucose utilization has been directly linked to the decrease in Na^+ cotransport into the astrocyte in these transporter-deficient mice [30].

16.1.1.2 Na^+/Cl^- -Dependent Neurotransmitter Transporters (SLC6). This family of transporters translocates substrates across the plasma membrane by coupling this process to the movement of Na^+ and Cl^- transport down their electrochemical gradients (Fig. 16.2c). Family members include the transporters for the inhibitory neurotransmitters GABA and glycine and the biogenic amine transmitters norepinephrine, dopamine, and 5-HT. Although we will not discuss this in detail, this family also contains the transporters for the osmolytes betaine and taurine, the amino acid proline, and the metabolic compound creatine [31]. Members of this family encode proteins of approximately 600 amino acids that have 12 TMDs, with N- and C-termini exposed to the cytosol and a large extracellular loop between TMDs 3 and 4 (Fig. 16.3c).

16.1.1.2.1 GABA Transporters (GATs). There are three GABA transporters, although a fourth, the betaine transporter, can also transport GABA. GAT1 [32] is the predominant GABA transporter in brain and is distributed widely. It is expressed at the highest density in the olfactory bulb, superior colliculus, substantia nigra, and magnocellular nucleus of the lateral hypothalamus; there is moderate expression in the hippocampus, cerebral cortex, pons, and granule layer of the cerebellum; its lowest expression is in cerebellar white matter, thalamus, and striatum [33]. Quantitative fluorescence indicates that GAT1 expression is, on average, on the order

of 1000 transporters/ μm^2 , with approximately 30–50% of them on the plasma membrane at any given time [33, 34]. GAT2 is found in meninges, ependyma, and choroid plexus. It does not overlap with markers of GABAergic signaling [35], and its interesting tissue distribution suggests that the transporter likely participates in regulating osmotic balance or GABA levels at the blood–brain barrier and in cerebrospinal fluid. GAT3 is found in neurons but is much more highly expressed in glia and its localization suggests a significant role in GABAergic signaling (Fig. 16.1a) [35].

The majority of functional data come from studies of GAT1. GAT1 likely functions as an oligomer [36]. Interaction between the fourth intracellular loop and the cytoplasmic N-terminus affects substrate translocation [37], while residues in the N-terminus affect the reorientation of the unliganded transporter [38]. The transport is electrogenic, with a stoichiometry of $2 \text{ Na}^+ : 1 \text{ Cl}^- : 1 \text{ GABA}$ (Fig. 16.2c). Chloride is required but does not contribute to the net charge transported per transport cycle and so may not unload during the cycle [39].

Even though the rate of GABA transport (tens of molecules per second) is too slow to respond to fast synaptic transmission, GAT1 is thought to provide binding sites for GABA, thus sequestering GABA from binding to GABA_A and GABA_B receptors [31]. In retina, reverse transport of GABA via GAT1 upon membrane depolarization contributes to the non-calcium-dependent release of GABA; calcium-dependent transmitter release may not occur in these cells [40]. In GAT1-deficient mice, the extracellular GABA level is increased. This results in an overactivation of GABA_A receptors and a postsynaptic tonic conductance. Moreover, chronically elevated GABA levels also downregulate phasic GABA release and reduce pre-synaptic signaling via GABA_B receptors, which results in an enhanced tonic and a diminished phasic inhibition [41]. Recently, it has been shown in cultures of midbrain neurons that currents mediated by the GABA transporter result in action potentials in these neurons. These transporter-initiated signals may be important in opiate withdrawal [42].

16.1.1.2.2 Glycine Transporters (GlyTs). Two isoforms of GlyT exist: GlyT1 is the predominant glial glycine transporter, whereas GlyT2 expression is restricted to neurons at glycinergic nerve terminals. The two isoforms have different stoichiometries: GlyT2 has a stoichiometry of $3 \text{ Na}^+ : 1 \text{ Cl}^- : 1 \text{ glycine}$, which predicts effective glycine uptake under all physiological conditions. In contrast, GlyT1 has a stoichiometry of $2 \text{ Na}^+ : 1 \text{ Cl}^- : 1 \text{ glycine}$, which suggests that the direction of glycine transport will be dictated by physiological conditions [43].

GlyT2 is the primary uptake site at inhibitory glycinergic synapses and thus likely regulates glycine signaling [44]. In addition to expression in glia at glycinergic synapses, GlyT1 is also expressed at glutamatergic synapses containing NMDA receptors. GlyT1 is involved in regulating glycine levels at the coagonist binding sites of these receptors [45]. GlyT1 knockout mice display severe motor deficits accompanied by lethargy, hypotonia, and hyporesponsivity. They usually die in the first day after birth due to respiratory failure, suggesting a role of GlyT1 in regulating centrally generated motor rhythms [46, 47].

16.1.1.2.3 Dopamine Transporters (DATs). In the brain, DAT distribution correlates well with dopaminergic innervation, with the highest density in striatum (including

putamen, caudate nucleus, and nucleus accumbens) and olfactory tubercle [48, 49]. Subcellularly, they are found in all neuronal processes and colocalize with markers for tyrosine hydroxylase and dopamine D₂ receptors [3]. The wide distribution of DATs beyond dopaminergic synaptic junctions suggests a role for DATs in regulating extracellular dopamine levels extrasynaptically [50].

DAT is a 620-amino-acid protein with a large hydrophilic extracellular loop between TMDs 3 and 4 containing two to four potential glycosylation sites (Fig. 16.3c). Dopamine is the major substrate for DAT, which also mediates uptake of norepinephrine, albeit inefficiently [51]. Other substrates include sympathomimetic amines, amphetamines, and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Cocaine is a high-affinity DAT antagonist. The stoichiometry of the dopamine uptake process is $2 \text{ Na}^+ : 1 \text{ Cl}^- : 1 \text{ dopamine}$, and therefore two positive charges are translocated with each dopamine molecule [52]. Such Na^+ influx accompanying inward transport of amphetamine (AMPH) is crucial for AMPH-induced dopamine efflux [53]. In addition to uptake, DATs also conduct an ion-channel-like inward current which results in an excitatory response in dopamine neurons at lower dopamine concentrations than that required for dopamine auto-receptor activation [54]. These DAT-associated currents may modulate excitability and regulate release of neurotransmitter from midbrain dopamine neurons.

Dopamine is involved in movement, cognition, attention, reward, goal-directed behavior, and blood pressure regulation [55]. DAT, which clears dopamine from the extracellular space, likely serves as an important regulator of signaling at dopaminergic synapses. Dopamine efflux through DAT contributes to autoinhibition of dopaminergic neurons [56]. In DAT knockout mice, dopamine persists at least 100 times longer in the extracellular space, leading to multifold increases in extracellular dopamine levels. These animals exhibit spontaneous hyperlocomotion and have cognitive deficits. Young homozygous DAT knockout animals gain weight slowly and show a significant propensity for premature death due to impaired food intake. Moreover, the ability of amphetamine and cocaine to elevate locomotor activity is abolished in these mice, even though self-administration of cocaine is preserved [57]. These results support a role for DAT in the behavioral action of addictive drugs. However, targets other than DAT likely contribute to the rewarding properties of cocaine in these mice [51].

16.1.1.2.4 Norepinephrine Transporters (NETs). NET is distributed widely in the central and peripheral nervous systems and is also found in a subset of adrenal chromaffin cells. In the adult brain, NET is confined to noradrenergic neuronal processes, including extensive arborizations within the hippocampus and cortex. Significant colocalization is seen with the axonal markers syntaxin, synaptophysin, and synaptosomal-associated protein (25 kD) (SNAP-25) [58].

NET is a 617-amino-acid protein that transports both norepinephrine and dopamine as well as other substrates, including sympathomimetic amines [59]. It is a major target of antidepressants and several psychostimulants. The stoichiometry of NET is $1 \text{ Na}^+ : 1 \text{ Cl}^- : 1 \text{ norepinephrine}$ and is therefore electrogenic [60]. Occasionally, NET switches to a channel mode during the transport cycle to allow a large influx of norepinephrine along with associated ions [61]. Structurally, N-glycosylation of NET is crucial for stabilizing surface expression but not for ligand recognition [62].

As with many members of this transporter family, NET is likely involved in termination of norepinephrine transmission. Consequently it is likely to regulate many central and sympathetic nervous system functions, including learning and memory, mood, attention, stress, and blood flow. NET knockout mice are viable and fertile but show reduced body temperature (1°C lower) and reduced body weight (20% less). These mice also display features similar to wild-type animals treated with antidepressants. Both cocaine and amphetamine are more effective in stimulating locomotion in NET knockout mice [63].

16.1.1.2.5 Serotonin transporters (SERTs). SERT is distributed widely in the central and peripheral nervous systems. In brain, SERT is highly concentrated in prefrontal cortex and raphe nuclei. Subcellularly, it resides at perisynaptic sites all along the axonal membrane, although somatodendritic uptake of 5-HT has also been observed [64, 65]. During development, SERT has also been found at glutamatergic thalamocortical afferents [66, 67]. Since the machinery to synthesize 5-HT is not present in these glutamate-containing neurons, it is suggested that thalamocortical afferents “borrow” 5-HT via transporter uptake and release it during the postnatal period in which SERT is expressed [67].

SERT, a protein of 630 amino acids, transports 5-HT as well as a variety of tryptamine derivatives. It is the primary target for several antidepressants and psychostimulants, including cocaine and (+)-3,4-methylenedioxymethamphetamine (MDMA, ‘Ecstasy’). The transport of 5-HT by SERT requires not only Na^+ and Cl^- but also countertransport of K^+ . The stoichiometry of transport suggests that transport should be nonelectrogenic [68]. However, channellike ion fluxes are observed in SERT both in the presence and absence of 5-HT [69], and SERT may switch from electrogenic modes to nonelectrogenic modes under different physiological conditions [70].

As its distribution indicates, SERT likely participates in serotonergic transmission and thus is involved in a variety of 5-HT-mediated behaviors, including mood, aggression, appetite, sleep, cognition, and motor activity. In SERT knockout mice, disruption of 5-HT uptake increases the extracellular concentration of 5-HT sixfold and reduces intracellular 5-HT by 60–80%. The amphetamine-induced locomotion-enhancing effect in these animals is also disrupted, although the ability to become addicted to psychostimulants is preserved [71]. Double-knockout mice lacking both DAT and SERT fail to show place preference for cocaine, indicating that both transporters are involved in cocaine-induced drug reward [72]. SERT and NET double-knockout mice show enhanced response to cocaine, suggesting a dopamine-independent role for norepinephrine signaling in cocaine action [73].

16.1.1.3 Choline Transporters (SLC5). Acetylcholine (ACh) is synthesized from choline and acetyl coenzyme A by the enzyme choline acetyltransferase (CHAT) in cholinergic nerve terminals. It is then transported into synaptic vesicles by the vesicular acetylcholine transporter (VACHT; see below) and released from the nerve terminal upon stimulation. After release into synaptic cleft, ACh is rapidly hydrolyzed into choline and acetate by the enzyme acetylcholinesterase. Choline is then taken up to presynaptic terminal for recycling by the choline transporter (CHT). This process is thought to be the rate-limiting step in ACh synthesis [74, 75].

CHT is expressed exclusively in cholinergic neurons and correlates very highly with the distribution of VACHT and CHAT. Subcellularly, CHT is highly concentrated at presynaptic cholinergic terminals. The majority of CHT expression is on small synaptic vesicles in the nerve terminal, with very little expression on the plasma membrane [76]. Thus CHT may only be plasma membrane resident during the time that vesicles containing ACh are fusing with the plasma membrane during neurotransmission. CHT is a high-affinity ($K_m < 10 \mu\text{M}$), Na^+/Cl^- -dependent transporter which belongs to the family of Na^+ -dependent glucose transporters. This family of transporters utilizes the Na^+ electrochemical gradient to drive the coupled transport of a variety of substrates (sugars, amino acids, vitamins, urea, and anions), as shown in Figure 16.2b. [77]. CHT, a 580-amino-acid protein, is predicted to have 13 transmembrane segments with an extracellular N-terminus and a large intracellular C-terminus (Fig. 16.3b) [78].

In CHT knockout mice, hemicholinium-3-sensitive choline uptake and subsequent ACh synthesis are specifically lost. Although morphologically normal at birth, homozygous CHT(–/–) mice become immobile, breathe irregularly, appear cyanotic, and die within an hour. Adult heterozygous CHT knockout mice overcome the reductions in CHT protein levels and sustain choline uptake activity through posttranslational mechanisms [79].

Traditionally, a transporter for ACh was thought unnecessary, given the high efficiency of acetylcholinesterase and the presynaptic reuptake of choline by high-affinity CHT. However, a recent study has documented the existence of a novel ACh transporter coded by the gene *snf-6* in *Caenorhabditis elegans*. Alignment of the predicted amino acid sequence of *snf-6* reveals 30–40% identity to members of the SLC6 transporter family [80]. Whether proteins of similar function also exist in mammals remains to be determined.

16.1.1.4 Glutamine Transporters (SLC38). As mentioned previously, glutamine is the precursor for synthesizing glutamate and GABA. Neuronally released glutamate is transported into astrocytes by plasma membrane glutamate transporters. Glutamate is converted to glutamine by glutamine synthetase. The glutamine must then be shuttled to neurons for amino acid synthesis. Glutamine is released from astrocytes in a nonexocytic manner and then picked up by the nerve terminal for use [81, 82]. Both processes are mediated by glutamine transporters, which belong to the family of Na^+ -dependent neutral amino acid (system N/A) transporters. The former process is mediated by the system N transporter on the plasma membrane of astrocytes. The latter process is mediated by the system A transporter on the plasma membrane of neurons [83, 84]. The system A and system N transporters each flux small, neutral amino acids, with a wider range of substrates for system A transporters. Each exhibit profound inhibition at low extracellular pH, which, in the case of system N subtypes but not system A, arises from the coupled countertransport of protons (Fig. 16.2d) [85]. Hydropathy analysis predicts a primary structure of 11 transmembrane segments, with an extracellular C-terminus and intracellular N-terminus. Between TMDs 5 and 6, there is a large loop exposed to the extracellular space [86] (Fig. 16.3d).

16.1.2 Vesicular Neurotransmitter Transporters

Neurotransmitters are synthesized in neurons and then must be concentrated in vesicles for subsequent Ca^{2+} -dependent release. The amount of transmitter

necessary for efficient synaptic transmission requires that each vesicle contain high-millimolar levels of neurotransmitter. Thus, vesicular transporters are absolutely required for normal brain function. However, vesicular transporters play other roles as well. The accumulation of neurotransmitter into vesicles acts as an amplification step for Na^+ -dependent uptake between the extracellular space and the cytoplasm as it increases the concentration gradient across the plasma membrane. Moreover, vesicular accumulation of neurotransmitter prevents the toxic effects that high transmitter concentrations might exert if present in the cytoplasm. Vesicular transport has been observed for all molecular weight transmitters, including ACh [87], the monoamines [88], glutamate [89], GABA [90], and glycine [91]. Transport by these carriers is driven by the proton electrochemical gradient ($\Delta\psi$) generated by the vacuolar ATPase that utilizes the energy of cytoplasmic ATP to translocate H^+ into vesicles and acidifies the organelle lumen (Fig. 16.2e) [92, 93]. Thus, all vesicular transporters are proton antiporters.

Based on sequence similarity and substrate binding, vesicular transporters can be divided into three families: the SLC17 transporter family for organic anions, including three vesicular transporters for glutamate (VGLUT 1–3), the SLC18 transporter family of vesicular amine transporters, including a VACHT and two for monoamines (VMAT 1–2), and the SLC32 transporter family for inhibitory amino acids, which includes vesicular transporters for GABA and glycine (VIAAT/VGAT). The structures of the vesicular transporter families are illustrated in Figure 16.4.

16.1.2.1 Vesicular Glutamate Transporters (SLC17). VGLUT 1 and 2 are localized exclusively in glutamatergic neurons of the brain and specifically at synaptic vesicles [94]. The distribution of VGLUT3 largely overlaps those of VGLUT 1 and 2. However, colocalization study indicates that VGLUT3 is also present in GABAergic interneurons, neuromodulatory neurons, and astrocytes. In addition to synaptic vesicles, VGLUT3 resides on vesicular structures of astrocytes and neuronal dendrites, suggesting novel roles for this transporter [95, 96]. The three types of VGLUT consist of 600-amino-acid residues and have been identified to contain 10 putative transmembrane domains with N- and C-termini exposed to cytosol (Fig. 16.4a). The three VGLUTs exhibit saturable glutamate transport with a K_m of approximately 1 mM [97, 98]. The stoichiometry of VGLUTs has not yet been determined but may vary for different isoforms [99, 100]. In contrast to other isoforms, VGLUT1 mediates a chloride conductance which could act to diminish $\Delta\psi$ and consequently regulate glutamate metabolism and synaptic release of transmitters [101].

16.1.2.2 Vesicular Acetylcholine and Monoamine Transporters (SLC18). VACHT is a unique marker for cholinergic synapses and neuromuscular junctions. It is coexpressed invariably with choline acetyltransferase [102]. Two isoforms of vesicular monoamine transporters exist: VMAT2 is found exclusively in monoaminergic neurons [103] while VMAT1 resides in neuroendocrine cells. Subcellularly, VACHT resides on small, clear synaptic vesicles of axon terminals that make symmetric contacts with dendrites [104]. VMAT2 is also expressed in small synaptic vesicles, but its major location in axon terminals is on large, dense core vesicles, which supports the role of large, dense core vesicles in the storage and release of monoamines [103]. Somatic and dendritic expression has also been observed for both VACHT and VMAT2, suggesting that these transmitters could be stored and released at these

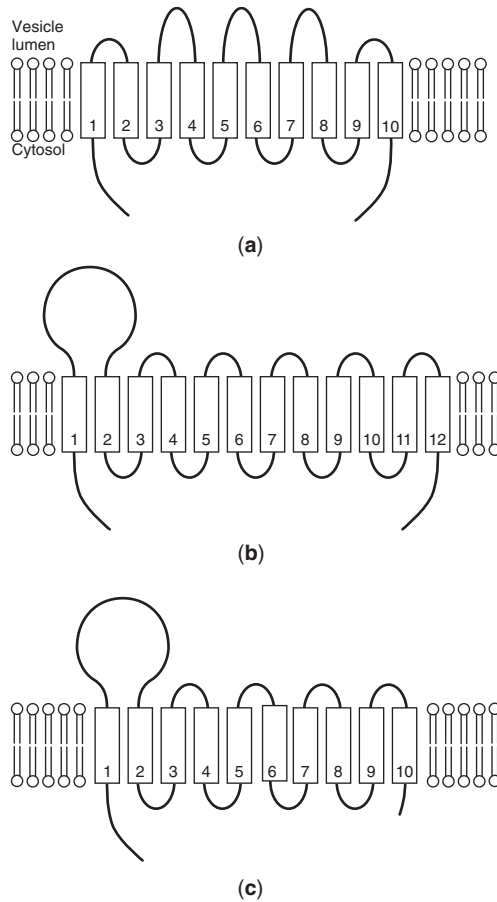


Figure 16.4 Schematic topology of vesicular neurotransmitter transporters: (a) SLC 17 (vesicular glutamate transporters); (b) SLC 18 (vesicular acetylcholine transporter and vesicular monoamine transporters); (c) SLC 32 (vesicular inhibitory amino acid transporters).

atypical sites [105]. Hydropathy analysis predicts 12 putative transmembrane segments, with a large hydrophilic loop between TMDs 1 and 2. This loop has been proposed to be inside the lumen of the vesicle, whereas both the N- and C-termini are likely to be cytoplasmic [106] (Fig. 16.4b).

VACHT and VMATs transport a single positively charged molecule into vesicles with a 2 proton : 1 amine stoichiometry. This predicts a transmitter accumulation into vesicles of approximately 500 mM [107]. Besides monoamines, VMAT2 transports dopaminergic neurotoxins such as 1-methyl-4-phenyl-phenylpyridinium (MPP⁺) from the neuronal cytoplasm into synaptic vesicles, from which amphetamines can induce their release.

Homozygous knockout of VACHT leads to failure of cholinergic neurotransmission in *C. elegans*, and homozygous knockout of VMAT in the worm leads to an egg-laying- and locomotion-defective phenotype similar to treatment with the VMAT inhibitor reserpine [108, 109]. VMAT2(−/−) animals exhibit marked deficits in locomotor activity and feeding behavior and die shortly after birth [110, 111]. These animals can

be partially rescued by amphetamine, which stimulates biogenic amine efflux through plasma membrane amine transporters. This is consistent with the observation that increased locomotor activity evoked by acute treatment with amphetamine is significantly higher in heterozygous VMAT2(+/-) mice than in wild-type mice [112]. The brains on the VMAT2 knockout mice show no gross morphological changes, and monoaminergic pathways do not appear altered [110, 112].

16.1.2.3 Vesicular Inhibitory Amino Acid Transporters (SLC32). VIAAT/VGAT is a neuronal vesicular transporter for both GABA and glycine. Therefore, the protein has been observed in GABAergic as well as glycinergic terminal boutons. Subcellularly, VIAAT/VGAT is restricted to small synaptic vesicles, as expected [113, 114]. Its putative structure predicts 10 TMDs, a long amino terminus and a short carboxyl terminus, and a large intraluminal loop between transmembranes 1 and 2 [115] (Fig. 16.4c).

VIAAT/VGAT transports GABA, glycine, and β -alanine into synaptic vesicles, with a stoichiometry of 1 proton : 1 amino acid [113]. The affinities of the transporter for GABA and glycine are approximately 5 and 25 mM, respectively. These low affinities may not be surprising given the relative abundance of these amino acids in the cytoplasm.

16.2 CLINICAL RELEVANCE OF NEUROTRANSMITTER TRANSPORTERS

Since vesicular transporters are responsible for determining the amount of transmitter released into the extracellular space and plasma membrane neurotransmitter transporters are essential for setting ambient levels of extracellular neurotransmitter, reduced or altered transporter function is likely to result in abnormal transmitter levels, improper cell signaling, and both acute and chronic pathophysiological states. Evidence relating neurotransmitter transporters to specific disorders comes from studies of transporter expression and function in human patients as well as studies of animal models and transporter knockouts.

16.2.1 Plasma Membrane Glutamate Transporters

Because glutamate is the most prevalent excitatory neurotransmitter in the brain, its regulation has an especially large impact on CNS function. Altered glutamate transporter expression and function are associated with a number of prevalent diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Huntington's disease, epilepsy, stroke, and glioma. The mechanism most often linked to these disorders is glutamate-induced excitotoxicity, which, as discussed above, could result from normal glutamate transporter function during pathophysiological conditions, or abnormal transporter expression or function.

A specific role for the glutamate transporter in human pathology has been most clearly elucidated for the motor neurodegenerative disease ALS. In humans, a majority of ALS patients show specific loss of EAAT2 in the motor cortex and spinal cord [116]. One possible explanation is that the loss of glutamate transporter protein is due to abnormally truncated RNA species, which have been observed in

many sporadic ALS patients. These RNA species are thought to have a dominant-negative effect, thereby suppressing EAAT2 protein expression [117]. However, it has been shown more recently that aberrantly processed mRNAs are also present in healthy individuals [118]. More recent work has identified a link to a mutation of the superoxide dismutase gene (*SOD1*), which is present in about 20% of ALS cases [3]. In transgenic mice with the mutant *SOD1* gene, EAAT2 expression is reduced by more than 90%, and the mice gradually develop hind-limb paralysis similar to that observed in the human disease. The mutant form of *SOD1* induces oxidative damage to the EAAT2 protein, suggesting that loss of EAAT2 in ALS could be due to oxidative conditions [116].

Although altered glutamate transporter expression has been associated with a number of neurological diseases and injuries, it is still unclear in many cases whether this change is a causative factor or a compensatory mechanism in response to increased glutamate levels. In stroke and ischemic/hypoxic brain injury, there is substantial evidence for a compensatory mechanism. A number of animal models of stroke have demonstrated that glutamate transporter expression increases following ischemia and hypoxia, suggesting that the expression of these transporters is altered in order to compensate for pathological conditions [119]. Also supporting this theory, brain damage in response to induced ischemia was exacerbated in mice expressing reduced GLT1 protein levels [120, 121]. However, several accounts conflict with these studies, instead reporting decreased glutamate transporter expression following ischemia [116].

In AD, the expression and function of EAAT1 and EAAT2 are altered. In postmortem studies of AD brains, reduced levels of EAAT2 were observed in frontal cortex [122]. This reduced expression was correlated with the presence of amyloid (A β) plaques, one of the trademark signs of AD. It has been suggested that the A β protein leads to glutamate toxicity by directly affecting glutamate transporter function. One possible mechanism is that A β instigates transporter reversal, thereby increasing extracellular glutamate to excitotoxic levels. Another possibility is that A β leads to the oxidation of the glutamate transporter and impedes transporter function, as in the *SOD1* mutant model of ALS [117].

Other disorders, such as Parkinson's disease (PD), have been shown to affect multiple neurotransmitter systems. While PD leads to the specific degeneration of dopaminergic neurons in the basal ganglia, glutamate as well as dopamine neurotransmission plays an important role in the disease. Compounds that alter glutamate transmission ameliorate parkinsonian symptoms in animal models of the disease [123, 124]. However, studies of glutamate levels in animal models of PD are inconclusive, with some studies reporting increased striatal glutamate concentrations and others showing no change. 3,4-Dihydroxyphenylalanine (L-DOPA) a common therapeutic approach in PD, has been shown to increase extracellular glutamate concentrations and glutamate transporter (GLT1) expression in the basal ganglia [125].

16.2.2 Plasma Membrane Monoamine Transporters

As described above, the plasma membrane transporters for dopamine, norepinephrine, and serotonin are targets for a number of clinical drugs and drugs of abuse. In addition, postmortem analysis and live imaging studies have established that changes in monoamine transporter expression are associated with several brain disorders.

Recently, specific gene polymorphisms for these transporters have been associated with increased risk of developing certain neurological disorders. These genes are currently being investigated for their capacity to cause changes in protein function or expression that may underlie disease.

16.2.2.1 Serotonin Transporters. SERT has been associated with affective disorders such as anxiety and depression as well as autism, substance abuse, and gastrointestinal disorders. The role of the serotonergic system in depression is well documented, with depressed patients exhibiting decreased plasma levels of serotonin as well as CNS changes in 5-HT receptors and transporters [126]. The class of antidepressants known as selective serotonin reuptake inhibitors (SSRIs) acts at SERT to prevent neurotransmitter reuptake and thereby increase extracellular 5-HT levels. The effectiveness of these antidepressants led researchers to question whether SERT dysfunction could be responsible for the decreased 5-HT levels observed in depression and other affective disorders. Supporting this idea, SERT is reportedly decreased in postmortem brain tissue from depressed patients [127]. Recent imaging studies in living subjects have reported a decrease in SERT availability in depressed subjects, although some studies have produced contradictory results [128, 129]. Additionally, two polymorphisms of the *SERT* gene are linked to increased likelihood of depression in humans; a variable number of tandem repeats (VNTR) located in intron 2 (5-HTT-VNTR) and a deletion/insertion in the promoter region (5-HTTLPR) [126, 130]. However, SERT polymorphisms are too rare to explain the prevalence of depression, and further research will be needed to establish the interaction of altered SERT expression and function with the other factors involved in depression and other mood disorders.

Several other affective disorders are linked to polymorphisms of the *SERT* gene. An uncommon single-substitution mutation of the *SERT* gene, *I425 V*, has been shown to contribute to autism, anorexia, Asperger's syndrome, and a familial form of obsessive compulsive disorder (OCD) [131]. This substitution leads to increased SERT function, thereby chronically reducing extracellular 5-HT levels [132]. Other alleles of the 5-HTT-VNTR polymorphism are associated with bipolar disorder and schizophrenia, although results vary across ethnic groups [133, 134]. We will discuss these monoamine transporter polymorphisms in more detail below.

5-HT is also found in peripheral systems, including platelets, the pulmonary endothelium, the placenta, and the gastrointestinal (GI) tract. It is released from gut endochromaffin cells to regulate fluid secretion and gut motility. Serotonin transporters are present on neurons and endothelial cells of the gut, where they prevent receptor desensitization by regulating extracellular 5-HT levels. SERT knockout mice exhibit impaired GI function. This may correspond to the GI problems that often accompany anxiety disorders and antidepressant treatments in humans [135]. SERT dysfunction could therefore be expected to lead to GI disorders. In agreement with this premise, the 5-HTTLPR is associated with increased instances of irritable bowel syndrome (IBS) [136]. In addition, SERT knockout mice show impaired GI motility [137]. However, as in the neurological disorders associated with SERT polymorphisms, IBS is influenced by a number of factors, and the importance of a single gene polymorphism among these factors is difficult to quantify.

16.2.2.2 Dopamine Transporters. The DAT is linked to PD, Tourette's syndrome, attention-deficit hyperactivity disorder (ADHD), and substance abuse. In all of these disorders, expression of DAT is altered, with decreases reported in PD, while ADHD and Tourette's disorder are characterized by increases in transporter density [138].

As in many disorders involving monoaminergic systems, ADHD seems to be regulated by multiple genetic and environmental factors. Both noradrenergic and dopaminergic systems appear to be affected in ADHD, as evidenced by the fact that stimulants used in ADHD treatment target DAT and NET [139]. Increased risk of ADHD is associated with a number of genes, including those for DAT, NET, and SERT [140]. In individuals with ADHD, increased expression of DAT has been observed [138, 141]. In a small subset of ADHD patients, a VNTR polymorphism of DAT appears to result in increased DAT expression in humans. Although this polymorphism is observed in only a small number of ADHD individuals, single-photon-emission computed tomography (SPECT) imaging shows substantially elevated DAT levels in these subjects [138]. Another line of research concerns the DAT knockout mouse, which has become an important animal model of ADHD. Like ADHD patients, DAT knockout mice show increased spontaneous activity and cognitive deficits and are calmed rather than excited by psychostimulants, an abnormality specific to ADHD [142]. However, in the mutant mice, overactivity is observed in novel situations but not in their home cage, whereas children with ADHD are generally quieter when exposed to new situations and more active in situations in which they are accustomed [141].

DAT plays an important role in PD, which is characterized by the death of dopaminergic neurons in the substantia nigra. This association was discovered when MPP⁺ (derived from MPTP) was shown to specifically poison dopaminergic cells after being taken up by DAT, reproducing the symptoms of PD [143]. This has led to speculation that environmental toxins could trigger PD via a similar mechanism [144]. Support for this theory comes from epidemiological studies showing a link between exposure to certain pesticides or other environmental toxins and the development of PD [145]. Thus far, genetic studies of the *DAT* gene show little association with PD [146].

16.2.2.3 Norepinephrine Transporters. Orthostatic intolerance is an autonomic system disorder that results in dizziness, syncope, fatigue, disordered thought, and postural tachycardia. Patients with this genetic disorder show plasma norepinephrine concentrations that are significantly elevated, and this raises the possibility that orthostatic intolerance is a deficit in NET clearance. Genetic analysis of the *NET* gene in these patients revealed a coding mutation that converts an alanine residue to proline in a TMD. Expression of a mutant NET protein carrying the proline site in transfected cells revealed a transporter with function that was less than 5% of wild type [147, 148]. This analysis revealed the first coding sequence mutant in a transporter associated with a disease state. More recently, it has been shown that this mutation not only disrupts the surface expression of the mutant but in coexpression with wild-type NET also prevents wild-type NET from trafficking normally [149].

16.2.3 Plasma Membrane GABA Transporters

Despite abundant physiological data to suggest that GABA transporters regulate GABAergic neurotransmission and the long practice of using GAT antagonists in the

treatment of epilepsy, the pathological link between these transporters and human diseases is not well established. This missing link is partly due to the complicated role that GABA transporters play in human diseases; GABA transporter deficiencies increase the susceptibility to hyperexcitability and thus may contribute to risk factors rather than directly causing symptoms.

GAT1 antagonists (i.e., tiagabine and NNC-711) [150–152] and glial (possibly GAT3-mediated) and other GABA uptake inhibitors (i.e., SNAP-5114, NNC 05-2045, and *N*-methyl-exo-THPO) [153–156] have neuroprotective and anticonvulsant effects. These are primarily mediated by elevating the ambient extracellular GABA concentrations.

Changes in GAT1 expression level are related to various neurological disorders. Upregulation of neocortical GAT expression is associated with seizure activity. In rat neocortex, hormone-induced seizures lead to an increase in GAT1-expressing interneurons [157] and a transient increase in GAT3 mRNA (but not protein) expression in seizure-induced rats 1 h after the last seizure [158]. Transgenic mice overexpressing GAT1 exhibit increased susceptibility to chemically induced seizures, although they do not display spontaneous seizure activity [159]. On the other hand, reduced GAT expression has been observed in the cortex of rat epilepsy models [160], in the neocortex of genetically epilepsy-prone rats [161], in the sensorimotor cortex of a rat pilocarpine model of temporal lobe epilepsy [162], and in the neocortex of patients with temporal lobe epilepsy and focal dysplasia [163]. This variation in GABA transporter regulation in epilepsy might reflect different pathophysiological mechanisms and temporal patterns of the disease states.

Following reports that the GABA uptake inhibitors tiagabine and NCC-711 exert neuroprotective effects, cortical expression of GAT1 and GAT3 was investigated in a rat model of transient focal ischemia [164]. Unlike GAT1, the expression of GAT3 was dramatically changed during this process. Neurons, including pyramidal neurons, became immunoreactive for GAT3. The novel reactivity of GAT3 at postsynaptic sites may increase GABA uptake, effectively reducing the amount of GABA available for binding to receptors and thus increasing neuronal excitability [164, 165].

16.2.4 Plasma Membrane Glycine Transporters

GlyT-deficient mice show phenotypes mimicking the symptoms of several human diseases. Newborn mice deficient in GlyT1 are anatomically normal but show severe motor and respiratory deficits and die within a day after birth [46, 47]. The deficits in GlyT1-deficient mice mimic glycine encephalopathy (also called nonketotic hyperglycinemia), a life-threatening disorder in neonates characterized by increased glycine levels in blood and cerebrospinal fluid accompanied by lethargy, hypotonia, and hyporesponsivity. Many patients become mentally retarded and/or develop seizures. Mice deficient in GlyT2 are normal at birth but during the second postnatal week develop a lethal neuromotor deficiency that resembles severe forms of human hyperreflexia (hereditary startle disease) and is characterized by spasticity, tremor, and an inability to right themselves [46, 47]. Although neither of the human glycine transporter genes has been linked to nonketotic hyperglycinemia or hyperreflexia, mutations in glycine receptors [166] and enzymes responsible for degrading glycine [167] have been implicated. It will be interesting to see whether dysfunctional glycine transporters also play a role in a subset of these conditions.

Since glycine is also known to act as a coagonist of excitatory NMDA receptors, it has been hypothesized that GlyT1 can influence excitatory synaptic signaling by regulating ambient glycine concentrations [45, 168, 169]. Placebo-controlled clinical trials with agents that directly or indirectly activate the glycine modulatory site consistently reduce negative symptoms and frequently improve cognition in patients with chronic schizophrenia who are receiving concurrent typical antipsychotics. Thus glycine transporters may be an important therapeutic target for the treatment of schizophrenia.

16.2.5 Vesicular Transporters

Thus far, only a few associations between vesicular transporters and disease have been described. VGLUT1 expression is decreased in the hippocampi of patients with schizophrenia [170]. It has also been proposed that the vesicular monoamine transporter (VMAT2) contributes to diseases of monoaminergic systems, including affective disorders, schizophrenia, PD, and other neuropsychiatric disorders. One piece of evidence for this idea is the finding that VMAT2 expression is decreased by exposure to clozapine, an antipsychotic drug that acts at serotonin receptors [171]. Vesicular transporters are likely to be important targets for therapeutic drugs. For instance, lobeline, a drug widely used as an emetic and to assist in smoking cessation, specifically targets VMAT2 and affects dopamine storage and release [172]. VMAT2 has also been targeted as a potential for gene therapy in PD [173].

16.2.6 Interactions Between Neurotransmitter Systems

Finally, many diseases involve multiple neurotransmitter systems and are influenced by a variety of genetic and environmental factors. A good example for this is schizophrenia, which is attributed in part to the dysfunction of dopaminergic systems. Dopamine release in this system is modulated by a number of neurotransmitters, including GABA, glutamate, acetylcholine, serotonin, and norepinephrine [174]. Accordingly, the prefrontal glutamatergic and GABAergic systems, including EAATs and GATs, have both been shown to undergo changes in schizophrenia. In fact, one hypothesis asserts that schizophrenia is caused not by dopaminergic problems but by dysfunction of cortical or subcortical glutamatergic transmission [175]. Altered glutamate transmission may be due to changes in VGLUTs or by altered levels of inhibitory transmitters [176]. A polymorphism of the *SERT* gene is also associated with greater risk of schizophrenia [134]. However, whether or not changes in various transporters are an underlying risk factor for schizophrenia, these transporters are currently being studied for their potential as targets of therapeutic drugs. For example, several new antipsychotic medications target the glycine transporter to inhibit uptake and thereby increase the amount of ambient inhibitory neurotransmitter [177]. The glutamate transporter has also been suggested as a possible target for drug intervention [178]. Because of the complicated modulation of dopamine neurotransmission, it is difficult to isolate any one of these systems as the cause of schizophrenia. However, the multiplicity of factors that contribute to dopaminergic transmission allow for a wide variety of therapeutic targets to be explored.

16.3 PLASMA MEMBRANE NEUROTRANSMITTER TRANSPORTER REGULATION

If fine tuning of phasic and tonic levels of neurotransmitter is crucial for normal cell signaling, then it is not surprising that the brain has developed mechanisms for fine tuning transporter action to achieve this goal. Changes in the activity or expression of transporters will have a significant impact on the amount of transmitter being released, the amount that reaches neurotransmitter receptors, how distant the transmitter can travel from its release site, and how long it remains extracellularly before it is sequestered. These changes, in turn, will influence pre- and postsynaptic responses during neurotransmission. In fact, endogenous mechanisms such as neural activity, hormones, growth factors, second-messenger systems, and posttranslational protein modifications regulate transporter expression and transport rates both acutely and chronically. These forms of regulation are, in general, likely to be homeostatic responses to sustain normal signaling; however, transporter dysregulation may impact several pathophysiological states as well. In the end, understanding the mechanisms by which transporters are regulated may provide insights into therapeutic approaches that target transporters. A complete examination of chronic and acute regulation of transporters is beyond the scope of this chapter. Instead, two examples of regulation will be examined in detail: chronic regulation of plasma membrane monoamine transporters and acute regulation of plasma membrane GABA transporters.

16.3.1 Chronic Regulation

16.3.1.1 Polymorphisms. Genetic polymorphisms in human transporter genes indicate individual mosaics in transporter expression level, cellular localization, uptake activity, and so on. Frequent linkages and associations of transporter polymorphisms with human diseases are discussed in the previous section. To understand how human transporter genes are organized and how their functions are regulated by polymorphisms is therefore of special interest from a clinical standpoint. Transporter gene polymorphisms include SNPs (single-nucleotide polymorphisms), polymorphisms in regulatory regions, VNTRs, Taq1 polymorphisms, variant splicing forms, and others.

16.3.1.1.1 Single-Nucleotide Polymorphisms. A site is considered polymorphic if the frequency of the observed allele is $> 1\%$ in human populations, while rare changes are termed mutations. Sequencing efforts have discovered at least 28 nonsynonymous coding SNPs in monoamine transporters (13 in NET, 5 in DAT, and 10 in SERT). In addition, synonymous coding SNPs, SNPs in introns, and SNPs in flanking regions have been found and have been deposited in the Single Nucleotide Polymorphism database at the National Center for Biotechnology Information (NCBI) (dbSNP) at <http://www.ncbi.nlm.nih.gov/SNP>. SNPs in noncoding regions do not directly affect the amino acid sequence of transporter proteins but may nonetheless contribute to transporter function alteration by changing their transcriptional and translational properties or by altering the targeting or trafficking of transporter proteins to appropriate cellular organelles. As mentioned in the previous section, one of the

SERT polymorphisms (I425 V) has been examined in detail and found to be a gain-of-function mutation that is refractory to regulation by protein kinase G [132].

16.3.1.1.2 Polymorphisms in Promoter Regions. Two polymorphic regions have been identified in human *SERT* gene promoter: 5'-HTTLPR and 5'-HTTSPR (long promoter region and short promoter region). The difference between these two variants is 44 bp, which is significant enough to induce a twofold serotonin uptake by the SERT with LPR [179]. Individuals with two short alleles, which are associated with decreased SERT expression, have a greater risk of anxiety, mood disorders, alcohol abuse, and various neuropsychiatric disorders, suggesting that such genetic regulation may contribute to disease [130].

16.3.1.1.3 Variable Number of Tandem Repeats. VNTR polymorphisms have been identified in monoamine transporter genes in introns such as the 3' untranslated region. As mentioned above, alleles of the 5-HTT-VNTR polymorphism are associated with bipolar disorder and schizophrenia, although results are different in specific ethnic groups [133, 134]. It is hypothesized that different numbers of copies of the repeat sequence might regulate the transporter function by affecting the mRNA stability, targeting properties, and translation efficiency.

16.3.1.1.4 Variant Splicing Forms. Primary transcripts from genomic DNA have splicing junctions where introns are excised and exons are connected to make coding DNA. By excising at different splicing sites, different coding DNA sequences may be generated from the same genomic DNA. Such regulation has been found in transporters and directly affects the transporter protein structure. For example, an alternatively spliced mRNA product of hNET having an additional leader sequence shows strong tissue-specific enhancement of transcriptional activity of the gene, thus increasing hNET expression [180]. Splice variants in the 3' region, which lacks certain protein-protein interaction sites, may result in the targeting of the protein to inappropriate cellular locations [181].

16.3.1.2 Multiple Transcription Initiation Sites. A number of canonical transcription binding sites found in the promoter region of SERT may be important in controlling the responses of transporter genes to regulatory factors. For example, a TATA-like motif, an AP1 site, a CREB binding element, AP2, NF-IL6, NF- κ B, and SP1 sites have been identified in the SERT promoter region [182]. The actual cellular signaling pathways responsible for initiating regulation are largely unknown.

16.3.1.3 Glycosylation. The large extracellular loop between TMDs 3 and 4 of plasma membrane neurotransmitter transporters contains consensus sites for post-translational glycosylation. In SERT, the absence of sugar moieties decreases the surface expression of the transporter without effect on steps related to 5-HT translocation [183]. The same is true for NET [184]. The loss in NET expression is correlated with a decrease in protein stability and half-life; however, these alterations do not account for all of the decrease in function in these mutants [62]. In DAT, glycosylation mutants are not excluded from insertion to the plasma membrane, but their time on the plasma membrane is significantly reduced [185].

Two more recent studies link glycosylation to two other aspects of monoamine transporter behavior. SERT is an oligomer [186], and in SERT mutants that are refractory for glycosyl modification, SERT monomers do not form oligomeric complexes. In addition, protein–protein interactions between SERT and myosin IIA are disrupted in these glycosylation mutants, which may affect the ability of SERT to be regulated by protein kinase G [187]. In NET, chronic exposure of the transporter to high levels of norepinephrine reduces the fraction of NET that is glycosylated. This downregulation in NET function may be associated with congestive heart failure [188].

16.3.1.4 Chronic Substrate Treatment. Since monoamine transporters are the sites of action of many clinically relevant drugs, some of which act as transportable substrates and others that are transporter antagonists, there has been much interest in the long-term regulation of transporter function by these and related compounds. In general, the compounds examined in detail include nontransporter substrates that act to alter transmitter levels, neurotransmitter receptor agonists and antagonists, and transporter substrates. For a more complete examination of this topic, see [189].

Multiday administration of reserpine, an inhibitor of VMAT, decreases NET [190] and DAT expression [191], while monoamine oxidase inhibitors, which act to prolong monoamine lifetimes, increases expression of these two transporters. Inhibition of the enzyme necessary for dopamine and norepinephrine synthesis downregulates DAT expression in brain, while administration of the dopamine precursor L-DOPA upregulates DAT expression [192]. Although the effects of these drugs over time are complex, as a first approximation, these findings are consistent with a homeostatic mechanism underlying chronic monoamine transporter regulation: Increased transmitter results in increased transporter expression and decreased transmitter results in decreased transporter expression. Consistent with this is the finding that activation of presynaptic adrenergic autoreceptors with clonidine, which downregulates norepinephrine release, reduces NET expression [193].

Less clear in terms of chronic regulation are the effects of drugs that target monoamine transporters directly. Some of this ambiguity is likely due to experimental systems being employed and the time course over which the experiments were performed. In animals treated with rather specific NET inhibitors, NET expression was either reduced [194] in some brain regions or not reduced at all [86]. In expression systems, the nonspecific antagonist and the nonspecific transportable substrate amphetamine also reduced NET expression [195]. For DAT, there is little consistent evidence for chronic regulation by DAT-specific substrates, cocaine or amphetamine; however, there is some evidence for short-term changes in expression during withdrawal from these compounds [189].

For SERT, there has been much interest in the long-term effects of SSRIs on transporter expression. In vivo, treatment of rats for three weeks with SSRIs results in a 50–80% reduction in SERT binding in multiple brain areas; this reduction is not due to a change in the number of 5-HT neurons [196]. In cultures of thalamocortical neurons that transiently express SERT, multiday treatment with SERT antagonists such as fluoxetine (Prozac) and cocaine reduce total SERT levels and the time course over which SERT is expressed. 5-HT and MDMA have the opposite effect [197]. Taken together, these studies again support the idea that cells regulate monoamine transporter expression in response to perceived transmitter levels. Since transporter

antagonists reduce transporter levels even in the presence of high extracellular levels of transmitter, this suggests that transporter activity may be in part the monitor that cells are using to determine how to adjust transporter levels.

16.3.2 Acute Regulation

Acute modulators regulate transporter function by changing both transport activity and/or transporter surface expression on a time scale of minutes. Over the past decade, significant progress has been made in defining the initiators of the regulation, the subcellular pathways that induce the regulation, and the mechanisms by which transporter function and expression are altered. While the majority of research in this field has been done on plasma membrane transporters of the SLC1 and SLC6 families, all transporters are subject to acute regulation. Readers are referred to a number of thorough examinations on this subject [189, 198–202]. Below, we give an example of the multiple forms of regulation by examining acute modulation of the plasma membrane GABA transporter GAT1.

16.3.2.1 Second Messengers. GAT1's primary sequence contains multiple consensus phosphorylation sites on serine and threonine residues [32]. Initial experiments [203] showed that drugs which alter PKC levels affect GABA uptake on a time scale of minutes. The magnitude and direction of PKC regulation are cell-type dependent [204, 205], but in neurons, PKC activation is correlated with a decrease in surface GAT1 expression and this change in transporter levels correlates well with changes in GABA uptake [206]. Indeed, PKC-mediated alterations in the subcellular distribution of transporter protein are a hallmark of all plasma membrane neurotransmitter transporters. Changes in surface expression could be due either to changing the rate of delivery of transporters to the cell surface, changing its rate of removal from the cell surface, or both. For GAT1, basal recycling rates are on the time scale of minutes. In the presence of PKC activators, the endocytosis rate increases twofold; the exocytosis rate is unaffected [34]. At least part of PKC's action on GAT1 is via direct phosphorylation of serine residues [207].

In neurons that endogenously express GAT1, tyrosine kinase (TRK) inhibitors decrease GABA uptake and tyrosine phosphatase inhibitors have the opposite effect. The decrease in uptake correlates with a decrease in direct tyrosine phosphorylation of GAT1 and results in a redistribution of the transporter from the cell surface to intracellular locations. Tyrosine residues in GAT1's first and third intracellular loops can be phosphorylated and are responsible for the regulation of GAT1 trafficking [208]. As with PKC, tyrosine kinase phosphorylation influences the rate of GAT1 internalization, but in the opposite direction. Interestingly, the relative levels of serine phosphorylation and tyrosine phosphorylation are negatively correlated. The data support the idea that GAT1 exists in either of two mutually exclusive phosphorylation states and that the relative abundance of these states determines in part the relative subcellular distribution of GAT1 [207].

In brain, these kinase pathways are likely activated by cell surface receptors. Specific agonists of G-protein-coupled receptors for acetylcholine, glutamate, and serotonin all downregulate GAT1 function [209]. This inhibition is mimicked by PKC activators and prevented by specific receptor antagonists and PKC inhibitors. TRK receptors, such as the receptors for nerve growth factor and brain-derived

neurotrophic factor (BDNF), induce tyrosine kinase activity. BDNF upregulates GAT1 function, and this increase correlates with an increase surface transporter expression [210].

A hallmark of neurotransmitter release is calcium- and depolarization-dependent exocytosis. This is also true of GAT1 [211]. In the presence of extracellular Ca^{2+} and depolarization, the amount of surface GAT1 increases. This increase is prevented in the absence of extracellular Ca^{2+} or in the presence of the Cd^{2+} , which blocks voltage-gated calcium channels. This suggests that many of the same factors that regulate transmitter release are also likely to regulate transporter expression.

16.3.2.2 Agonists and Antagonists. As discussed in the section on chronic regulation, if cells regulate transporter expression in order to control extracellular transmitter levels, then the most direct route would be to have the transporter directly sense transmitter levels and regulate its expression. Specific GAT1 substrates upregulate transport; GAT1 transport inhibitors that are not transporter substrates downregulate transport. Increases in transport are correlated with an increase in surface transporter expression, which results from a slowing of GAT1 internalization [212]. Incubation of GAT1-expressing neurons with transporter ligands alters the amount of GAT1 that is tyrosine phosphorylated, suggesting a model in which substrates permit the phosphorylation of GAT1 on tyrosine residues and that the phosphorylated state of the transporter inhibits transporter internalization [208].

16.3.2.3 Interacting Proteins. The ability to regulate transporter trafficking suggests that the machinery involved in trafficking will interact with transporters. Overexpression of the plasma membrane SNARE proteins [213, 214] syntaxin 1A or SNAP-25 alters the subcellular distribution of GAT1 [201] and toxins that specifically inactivate these proteins regulate GAT1 endogenously in neurons [215]. Syntaxin 1A acts as a positive regulator of GAT1 surface expression. Immunoprecipitation experiments reveal a physical interaction between GAT1 and syntaxin 1A [206] and the association of GAT1 with syntaxin 1A is mediated predominantly by amino acids 30–54 in GAT1's N-terminal cytoplasmic tail [216]. Syntaxin-1A-regulated trafficking effects are also seen for GlyT [217], NET [218], and SERT [219, 220].

Regulating transporter trafficking represents one method for modulating extracellular transmitter levels and cell signaling. Another way is to directly alter rates of transmitter transport. Measurements of flux rates show that syntaxin 1A reduces GABA transport from seven to approximately two molecules per second [216]. Application of transporter substrates increases the flux rate through GAT1, essentially reversing syntaxin 1A inhibition [221]. The substrate-induced rate change requires the presence of syntaxin 1A, and in neurons that endogenously express GAT1 and syntaxin 1A, substrate application decreases the fraction of syntaxin 1A that is bound to GAT1 on a time scale comparable to the substrate-induced change in flux rates. These data suggest that substrate translocation regulates GAT1–syntaxin 1A interactions and provide a mechanism by which GABA transport can be increased during times of rising synaptic GABA concentrations. Furthermore, these and other data suggest that many of the processes involved in transporter regulation interact to ultimately determine transporter function and ultimately neural function.

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GASEOUS SIGNALING: NITRIC OXIDE AND CARBON MONOXIDE AS MESSENGER MOLECULES

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17.1	Introduction	743
17.2	Physiological Role of NO	745
17.3	Nitrosylation as Signaling Mechanism of NO	745
17.4	Detection of Nitrosothiols	747
17.5	Effects of Nitrosylation in Cellular Pathways	748
17.5.1	Ion Channels	748
17.5.2	Protein-Protein Interactions and Nitrosylation	749
17.5.3	Gene Transcription	750
17.5.4	Vesicular Transport	751
17.5.5	Extracellular Matrix	751
17.5.6	Cell Survival	752
17.6	Physiological Function of S-nitrosylation	752
17.7	Nitric oxide and Neurodegeneration	754
17.8	Nitrosylation and PD	755
17.9	Carbon Monoxide as Signaling Molecule	756
17.10	Concluding Remarks	757
	Acknowledgments	757
	References	757

17.1 INTRODUCTION

Nitric oxide (NO) is one of the major components of air pollution related to modern human activities. In biological systems, NO was first described as an endothelium-derived relaxing factor (EDRF) because of its action on relaxing smooth muscle in blood vessels. Soon it was found that NO can regulate pathways ranging from hormonal control in plants to blood circulation and neurotransmission in mammals. In tissues, endogenous NO is synthesized by nitric oxide synthases (NOSs) that

convert L-arginine to NO and L-citrulline [1]. There are at least three classes of NOS in animals—neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS)—and they are responsible for producing NO in different tissues (Fig. 17.1). Nitric oxide synthases produce NO by oxidizing L-arginine to L-citrulline and NO with the use of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (Fig. 17.2). NO is an important signaling molecule that can control a variety of processes, and in fact many pharmacological targets are aimed at the NO signaling pathways. One of the best examples is nitroglycerin. Nitroglycerin has been used as a therapeutic agent for relieving symptoms of cardiovascular diseases for more than a century, even before NO was identified as the EDRF in the 1980s. Another gas molecule that is emerging as a signaling agent is carbon monoxide (CO). It has also been suggested that NO and CO might coordinate together to mediate different signal transductions [2]. The in-depth understanding of the molecular signaling mechanisms of these gaseous signaling molecules should provide valuable information in the development of new pharmacological targets for different diseases.

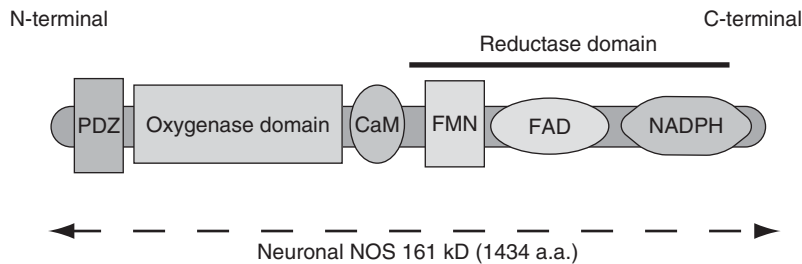


Figure 17.1 Structural domains of neuronal nNOS. The enzyme is made up of 1434 amino acids with several conserved domains. nNOS contains a post-synaptic density protein (PSD95), a Drosophila large tumor suppressor (DlgA) and azol (PDZ) domain, an oxygenase domain, a calmodulin binding domain (CaM), and a reductase domain. Within the reductase domain, there are conserved domains that can bind to flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH. Both eNOS and iNOS consist of a similar domain structure except for the absence of the PDZ domain. (See color insert.)

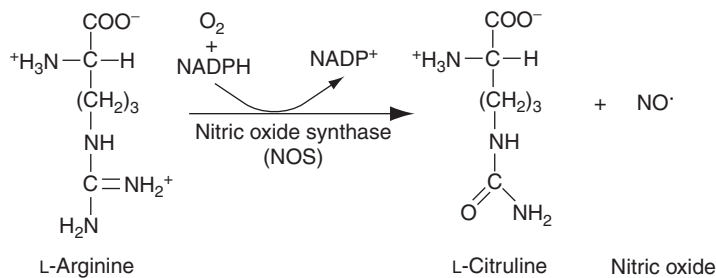


Figure 17.2 NOS converts L-arginine to L-citrulline and NO in the presence of NADPH and oxygen.

17.2 PHYSIOLOGICAL ROLE OF NO

Since the discovery of NO as an important signaling molecule, a number of physiological functions were found to be modulated by NO. The most well known effect of NO is its blood vessel dilating activity. NO originating from endothelium was first described as an EDRF because of its action on relaxing smooth muscle in blood vessels. NO from the endothelium is produced by eNOS in response to different stimuli such as shear force generated from blood flow. The NO generated from the endothelium can then diffuse to the smooth muscle of blood vessels and activate soluble guanylate cyclases (GCs) to produce cyclic guanosine monophosphate (cGMP), which can subsequently induce vessel dilation. eNOS knockout mice suffer from consistent hypertension, which supports the importance of eNOS in the regulation of the vascular system [3]. In the central nervous system, NO functions as a neurotransmission modulator. For instance, the PDZ domain of nNOS is linked to the *N*-methyl-D-aspartic acid (NMDA) receptor through postsynaptic density protein 95 (PSD95) where it modulates NMDA receptor neurotransmission [3]. In the immune system, macrophages use NO produced by iNOS to kill microbes. The activation of iNOS during the inflammatory response is also responsible for NO-induced tissue injuries in the autoimmune diseases, such as multiple sclerosis (MS) [4]. Excessive production of NO in the brain promotes neuronal toxicity under unfavorable conditions. For example, during cerebral ischemia increased stimulation of excitatory neurotransmitter glutamate receptors will result in the influx of Ca^{2+} and activation of nNOS to increase the production of NO [5]. The high levels of NO can in turn react with reactive oxygen species to form the highly toxic peroxynitrite and promote neuronal cell death [5]. Interestingly, in ischemic preconditioning, NO activates p21Ras, which can promote neuronal survival against subsequent major ischemic challenge [6]. Some of the mechanisms of how NO can mediate such a diverse spectrum of physiological functions is still not fully understood. However, it is emerging that nitrosylation of metal or cysteine residues in proteins might represent one of the major mechanisms of how NO can achieve its role in signal transduction in different systems.

17.3 NITROSYLATION AS SIGNALING MECHANISM OF NO

NO is a highly reactive free-radical molecule that has an unpaired electron occupying an antibonding π orbital [7, 8]. The unique chemical properties of NO facilitate its role as an important signaling molecule in biological systems. NO can mediate its effect by reacting with biological molecules directly or through the help of other reactive oxygen species [8] (Fig. 17.3). For instance, NO reacts with ferrous ion containing heme complexes such as in guanylate cyclase (GC), P450, and in hemoglobin to form an iron nitrosyl complex in that mediates functional changes in these proteins [8, 9]. The activation of GC by NO is a good example of how NO functions as a signaling molecule through the direct modification of biological molecules. Guanylate cyclases are enzymes that convert guanosine triphosphate (GTP) to cGMP [10]. NO activates GC to increase cGMP levels [10]. In the basal and inactivated state of GC, the heme iron and the axial ligand formed from the histidine residue in the enzyme prevent the active site of GC from being easily accessible by

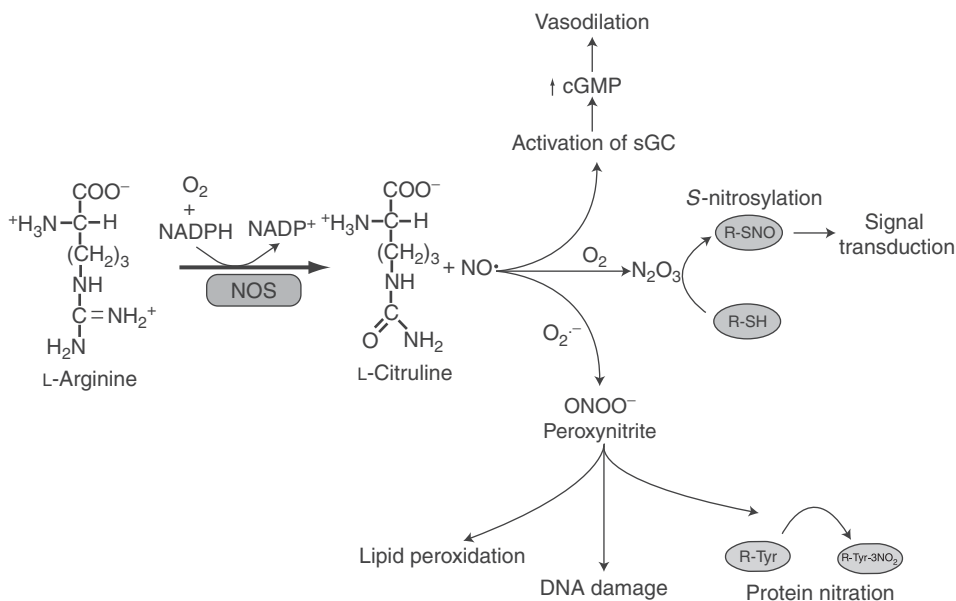


Figure 17.3 Schematic diagram showing how NO can react with different biological molecules to mediate its effect. NO produced from NOS can activate directly sGC to promote vasodilation in blood vessels via increasing production of cGMP. NO can S-nitrosylate cysteine residues in proteins to mediate its effect on signal transduction. NO can also react with superoxide anion and induce damage on various cellular components. (See color insert.)

substrate [10]. NO activates GC by binding to the heme group in GC, which results in a conformational change of the enzyme making the active site more available to its substrate [10]. The increased production of cGMP from GC can then activate different effectors to mediate cellular responses such as vasodilatation in blood vessels.

Oxygen and other free radicals can also react with NO to form reactive nitrogen oxide species and to exert its effects on different biological molecules [7–9]. For example, superoxide anion can react with NO to form peroxynitrite. Highly reactive peroxynitrite can then damage protein by reacting with the tyrosine residues to form 3-nitrotyrosine, induce lipid peroxidation, and cause DNA damage. Another major signaling mechanism of NO is the formation of nitrosothiols through the process of nitrosylation. Nitrosylation refers to the attachment of a NO molecule to a metal ion or cysteine residue in proteins. The reaction is reversible and needs to be distinguished from nitration, which refers to the introduction of a NO_2 group to a tyrosine or tryptophan residue in protein. An increasing number of proteins are modified by NO through nitrosylation, and the modification is viewed as an important redox sensing and/or signaling mechanism that may be comparable to phosphorylation [11]. One of the characteristics of nitrosylation is its reversibility and the dependence of a specific acid–base motif in the protein structure [12, 13]. The initial discovery of the acid–basic motif requirement was based on the comparison of primary sequences of known nitrosylated proteins. It was soon found that cysteine residues that are preceded by an acidic amino acid and then followed by a basic amino acid have a

significant higher probability for being modified by NO through *S*-nitrosylation. However, it was later observed that the acid–base motif is not necessarily constrained to a primary sequence. For example, cysteine residues 221 and 273 in dimethylargininase-1 (DDAH-1) has been shown to be modified by NO through *S* nitrosylation [14]. Surprisingly, it was found that no acid–base motifs are flanking the primary sequence of these cysteine residues [14]. With a more detailed three-dimensional structural simulation analysis, it was found that these cysteine residues are actually surrounded by acidic and basic amino acids that are brought together by the tertiary structure and can therefore form the acid–base motifs to facilitate the process of *S* nitrosylation [14].

The mechanisms of how proteins are nitrosylated and denitrosylated are not well characterized. It has been speculated that *S*-nitrosylation might require actions of certain enzymes to facilitate the process, but so far only metabolism of *S*-nitrosoglutathione (GSNO) to *S*-nitrosocysteinyl glycine (CGSNO) by γ -glutamyl transpeptidase (γ -GT) is found to facilitate the process of nitrosylation [15]. In general, the formation of nitrosothiols is believed to involve the reaction of NO and O₂ to form the N₂O₃ as a first step that can subsequently react with thiol groups in proteins to form the resultant nitrosothiols [11]. The metabolism of nitrosothiols is also not fully understood. Components in cells are known to be able to metabolize nitrosothiols, and at least one enzyme, glutathione-dependent formaldehyde dehydrogenase (GS-FDH), has been identified to have high affinity to metabolize GSNO. GS-FDH is highly conserved from bacteria to humans and the enzyme is now referred to as *S*-nitrosoglutathione reductase (GSNOR) [16]. Since GSNO is regarded as the reservoir of nitrosothiols, regulation of its levels by GSNOR might represent an important metabolic control of nitrosylation in proteins [15, 16]. It is interesting that GSNOR only has high affinity to GSNO; other nitrosothiols such as *S*-nitrosocysteine or *S*-nitrosohomocysteine, are not affected by this enzyme, which suggests that other enzymes might also contribute to the overall metabolism of nitrosothiols [16].

17.4 DETECTION OF NITROSOTHIOLS

A number of methods are available to detect *S*-nitrosylation, but they both have their advantages and disadvantages [11]. Therefore a number of methods are usually employed to study *S*-nitrosylation.

The detection of nitrosothiols by chemiluminescence is one of the most sensitive approaches to measure *S*-nitrosylation. It was first developed to measure nitrosothiols in plasma [17] and later found to be able to distinguish different derivatives of NO species [18]. Generally, the basic idea is to release NO from nitrosothiols by ultraviolet (UV) radiation or chemical methods. The released NO is then reacted with ozone and the resultant chemiluminescence signal is measured. Despite the sensitivity, this method requires substantial investment of instruments and is usually not available in most laboratories.

Electrospray ionization mass spectrometry (ESI-MS) has been used with success in studies to detect *S*-nitrosylation in some proteins [14, 19, 20]. Apart from being able to detect *S*-nitrosylation in protein, ESI-MS can also provide stoichiometric information regarding the modification. Usually, a purified protein is exposed to an NO donor and the resultant *S*-nitrosylated species will be digested by trypsin and the

NO-modified peptide will be detected and identified by ESI-MS. Up to now, only ESI-MS has been proven to be able to detect *S*-nitrosylation *in vitro*; the more widely available matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was found to destroy the modification during the process of ionization [19].

Colorimetric and fluorometric methods remain one of the most commonly used approaches to detect nitrosothiols [21–23]. These methods use metal ions such as Hg^{2+} or Cu^{2+} to release the NO from samples containing nitrosothiols. The released NO can subsequently react with chemical reagents to form a colored or fluorescent compound [24]. The colored or fluorescent compound can then be measured by a spectrophotometer to quantify the amount of nitrosothiols in the samples. These methods are simple to perform with common laboratory equipment. The fluorometric method is usually more sensitive than the colorimetric method, but both methods suffer from potential interference from protein or biological molecules in the samples [24].

A more versatile approach to label *S*-nitrosylated proteins with a biotin moiety [25], the so-called biotin-switch method, can be used to detect *S*-nitrosylation in both *in vitro* and *in vivo* conditions. Essentially, the method includes the initial blocking of free cysteines in a nitrosylated protein with methyl methanethiosulfonate (MMTS) and then NO is released from the S–NO group by ascorbate. The free cysteine generated is subsequently labeled with biotin by a reaction with *N*-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP). The labeled protein can be purified by avidin agarose for Western blot analysis or proceed for proteomics analysis. Although this method has been proven to be effective in detecting a wide range of *S*-nitrosylated proteins *in vitro* and *in vivo*, the sensitivity of this method is not as robust compared with other methods. The biotin-switch method also cannot be used to quantify the amount of nitrosothiols in samples.

Antibodies that were raised in rabbits by using *S*-nitrosocysteine as an antigen have been used in immunohistochemistry or Western blotting to detect *S*-nitrosylated proteins with success [22, 26, 27]. Although it is a convenient method to detect nitrosothiols in biological tissues, the antibodies tend to cross react with unmodified cysteine residues in protein, and therefore multiple controls are usually required with the use of these antibodies. For example, treating the sample with Hg^{2+} to release the NO can be used as a negative control, and a positive control can be performed by treating samples with an NO donor such as GSNO.

17.5 EFFECTS OF NITROSYLATION IN CELLULAR PATHWAYS

17.5.1 Ion Channels

Both ryanodine and NMDA receptors (NMDARs) are known to be regulated by NO through *S*-nitrosylation. The ryanodine receptor is a calcium channel that is highly expressed in skeletal muscle and is responsible for the release of calcium from the sarcoplasmic reticulum during muscle contraction [28, 29]. Activity of the ryanodine receptor is sensitive to changes in O_2 tension. The *S*-nitrosylation of 6–8 out of 50 cysteine residues in the receptor is responsible for activation of the channel in response to changes in O_2 tension [28, 29]. At least five cysteine residues in NR1 and NR2A subunits of NMDARs are modified by NO through *S*-nitrosylation [13, 30].

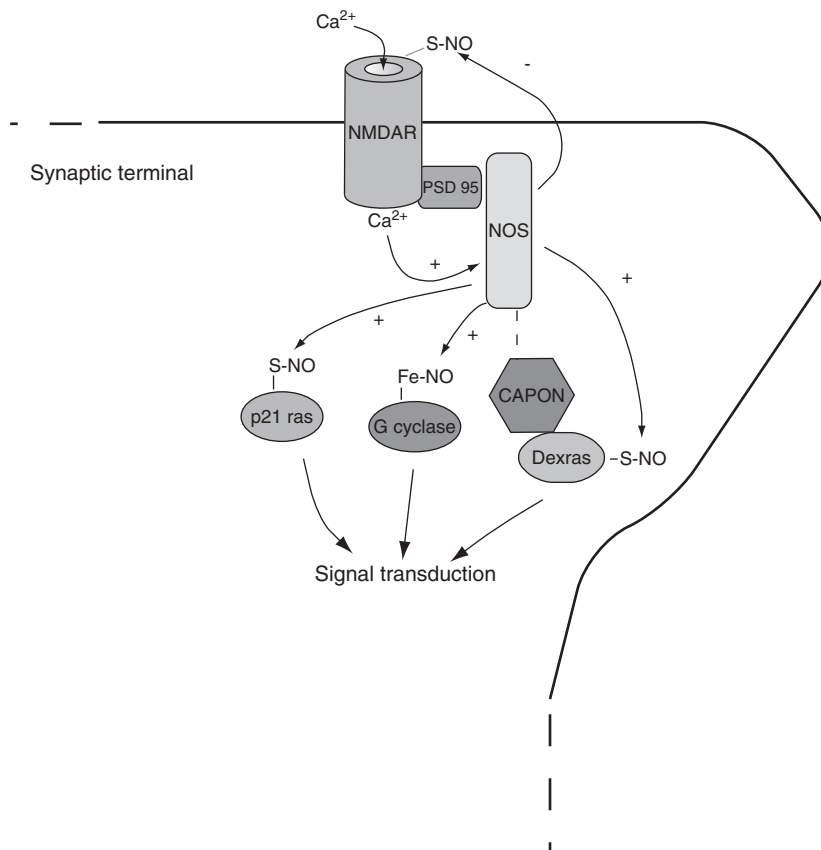


Figure 17.4 Schematic illustration of NMDAR complex signaling regulated by NO through *S*-nitrosylation. Ca^{2+} influx from the activation of NMDAR promotes the production of NO from the nearby nNOS. NO can then activate downstream signaling cascades such as p21Ras, GC, and Dexras. NO can also inhibit the activity of NMDAR through *S*-nitrosylation, which serves as a negative-feedback mechanism for the receptor complex [13]. (See color insert.)

In particular, nitrosylation of cysteine residue 399 of NR2A significantly inhibits the activity of the receptor [30]. The organization of the NMDAR complex also facilitates the control of its activity through nitrosylation. Since, nNOS is linked to the NMDAR complex via the PDZ domain, Ca^{2+} influx resulting from the activation of the receptor can activate nNOS to produce NO. The increased NO production can then act through *S*-nitrosylation and provide a feedback mechanism for the NMDAR complex [30, 31] (Fig. 17.4). Other downstream signaling molecules proximate to the receptor such as p21Ras and Dexras can all be modulated by *S*-nitrosylation [25, 32, 33] (Fig. 17.4).

17.5.2 Protein–Protein Interactions and Nitrosylation

There is always an intense interest to understand the mechanism of how *S*-nitrosylation can mediate its effect. Apart from modulating the enzymatic activities through the *S*-nitrosylation of critical cysteine residues in the active sites, another

emerging mechanism is to interfere with or promote protein–protein interactions when proteins are *S*-nitrosylated. For instance, in eNOS, dimerization of the enzyme is required for its activation. Interestingly, *S*-nitrosylation of eNOS can selectively interfere with the dimerization of eNOS, which can function as a negative-feedback mechanism [34]. In Hdm2, a human homolog of Mdm2, nitrosylation of Cys77 selectively interferes with the binding of its substrate, p53 [35]. In a recent report, a modified yeast two-hybrid screening was used to screen for protein–protein interactions that is dependent on nitrosylation. In this screening, it was found that interaction of procaspase-3 with acid sphingomyelinase (ASM) and NOS are NO dependent [36]. The *S*-nitrosylation of procaspase-3 selectively enhances its interaction with ASM and the interaction seems to inhibit basal caspase activity [36]. The interaction between procaspase-3 and NOS is dependent on the enzymatic activities of NOS. This finding also explains why cells expressing NOS may be resistant to cell death stimuli such as tumor necrosis factor and cyclohexamide [36].

17.5.3 Gene Transcription

NO is known to be involved in signaling cascades required for gene transcription. One of the mechanisms is through the modulation of the transcription factors, hypoxia-inducible factor (HIF), p53, and nuclear factor kappa B (NF- κ B) [37]. For instance, a recent paper has revealed how NO can regulate the activity of NF- κ B [38]. Under normal conditions, NF- κ B is sequestered by inhibitory κ B (I κ B) to the cytoplasm, and this prevents it from entering into the nucleus to initiate transcription. When the pathway is activated, I κ B kinase (IKK) phosphorylates I κ B and promotes I κ B degradation through the ubiquitin pathway [39]. The downregulation of I κ B allows NF- κ B to translocate to the nucleus and activate gene transcriptions [39]. IKK is subject to redox regulation, but the mechanism is not clear. On the other hand, NO is known to have an inhibitory effect on the NF- κ B activated transcription, and it has long been suspected that NO might mediate this inhibition via IKK. In fact it was found that NO can inhibit the kinase activity of IKK through *S* nitrosylation of Cys-179 of the beta subunit of IKK, thus inhibiting the activation of NF- κ B [38].

The mechanism of how NO affects the HIF-1-mediated transcription is not fully understood, but NO promotes the accumulation of HIF-1 in the nucleus and hence activates HIF-1-induced gene transcriptions [40]. The complex control of HIF-1-induced gene transcription in response to hypoxia is regulated in part through ubiquitination. Under normal normoxic condition, HIF-1 is hydroxylated by prolyl hydroxylases (PHDs) so that HIF-1 can be targeted by ubiquitin E3 ligase, pVHL (von Hippel Lindau protein), for degradation through the ubiquitin proteasomal pathway [37, 40]. On the other hand, during hypoxia, low O₂ level prevents PHD from hydroxylating HIF-1, resulting in the accumulation of HIF-1 in the cytosol [37, 40]. This leads to the translocation of HIF-1 into the nucleus, promoting the transcription of hypoxia-responsive genes [37, 40]. More detailed study suggests that NO acts on PHD and attenuates its activity on HIF-1, which ultimately promotes the accumulation of HIF-1 in the nucleus [40]. The mechanism of how NO can inhibit PHD is not clear, but it is highly possible that such action is mediated through nitrosylation [37].

Apart from interfering indirectly with gene transcription pathways, *S*-nitrosylation has also been found to modify transcription factors directly. For instance, the binding of estrogen receptor (ER) to the estrogen-responsive elements (EREs) can be directly interfered by NO [41]. This interference has been shown to affect the ER-mediated gene transcription in cells [41]. NO appears to selectively *S*-nitrosylate cysteine residues that coordinate Zn^{2+} in the two major DNA binding domains of ER and hence directly prevent it from binding to EREs in the activation of ER-dependent gene transcriptions [41].

17.5.4 Vesicular Transport

Vesicular trafficking is a critical cellular process that regulates many physiological functions. The process is mediated through the action of the SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor) complex. For example, exocytosis is mediated by the formation and transition of the *trans*-SNARE complex to the *cis*-SNARE complex during the fusion of vesicles to the cytoplasmic membrane [42]. Another component of this process is the *N*-ethylmaleimide-sensitive factor (NSF). NSF is an adenosine 5'-triphosphatase (ATPase) that promotes the dissociation of the stable *cis*-SNARE complex so as to recycle components during exocytosis [42]. NO is known to inhibit vascular inflammation, possibly through the inhibition of exocytosis of inflammatory granules (Weibel–Palade bodies) in endothelial cells that mediate vascular inflammation and thrombosis [43]. It is now clear that NO inhibits exocytosis of Weibel–Palade bodies through the *S*-nitrosylation of Cys-91 and Cys-264 on NSF [21]. Interestingly, nitrosylation of NSF does not affect its ATPase activity but instead prevents it from disassembling the stable *cis*-SNARE complex during exocytosis [21]. These results suggest that those cysteine residues might be important for NSF to transfer chemical energy from ATP to the mechanical energy required for the disassembly of the SNARE complex [21].

17.5.5 Extracellular Matrix

The extracellular matrix is regulated in part by matrix metalloproteinases (MMPs). MMPs are a family of endopeptidases that degrade the extracellular matrix and have been implicated in a number of diseases [44]. For example, MMP-1 was found to function as a protease agonist of the protease-activated receptor 1 (PAR1), and its signal contributes to the metastatic processes of breast carcinoma [45]. Another MMP, MMP-9, has been found to be elevated in patients after stroke [46]. The important role of MMP-9 in stroke is confirmed by its activation in a mouse stroke model using focal cerebral ischemia and reperfusion. In mice pretreated with MMP inhibitors before cerebral ischemia and reperfusion, the cerebral infarct size was consistently reduced, which supports the importance of MMP-9 activation during ischemia [47]. Interestingly, activation of MMP-9 during ischemia has been recently attributed to *S*-nitrosylation [47]. MMP-9 was found to be activated by *S*-nitrosylation of a cysteine residue in the propeptide domain that coordinates Zn^{2+} in the active site [47]. Activation of MMP-9 seems to have a deleterious effect on neuronal survival. For instance, treatment of neurons with NO-activated MMP-9 increases the rate of apoptosis, and this effect can be reversed by the MMP inhibitor, GM6001 [47]. In the mouse stroke model, activation of MMP-9 activity by focal cerebral

ischemia can be abrogated by the treatment of animals with the specific nNOS inhibitor 3-bromo-7-nitroindazole (3br7NI). Taken together, these results provide insights into how extracellular matrix proteolysis is related to neuronal cell death through *S*-nitrosylation of MMP-9 during ischemia [47].

17.5.6 Cell Survival

NO is well known for its dual effects on cell survival. Both proapoptotic and antiapoptotic pathways have been linked to the action of NO, but how it can mediate such dramatic effects on the cell survival and cell death is still not completely understood. *S*-nitrosylation seems to play an important role in some apoptotic pathways. For instance, both caspase-3 and caspase-9 are *S*-nitrosylated in the mitochondria but only caspase-3 is *S*-nitrosylated in the cytoplasm [48]. Nitrosylation of caspase-3 on the catalytic site cysteine inhibits apoptosis in cells [48, 49]. In Fas-induced apoptosis, one of the actions of Fas is to induce the denitrosylation of caspase-3, supporting the important role of nitrosylation in modulating cell death [49]. In redox-regulated cell survival, NO mediates its effect through the nitrosylation of thioredoxin [22]. Thioredoxin is well known to regulate cell growth, in part by inhibiting apoptosis [50]. In endothelial cells, thioredoxin mediates its function, in part by maintaining the content of *S*-nitrosothiols, and also contributes to the antiapoptotic function [22]. Thioredoxin itself also is nitrosylated at cysteine residue 69, and this modification is necessary for its protective function [22]. Another protein that is well known to be modified by NO through *S*-nitrosylation and to affect cell survival is p21Ras. The Ras GTPase is a small protein that regulates important signaling cascades in cell survival or cell death [51]. The Ras protein functions as a molecular switch that alternates between a GDP-bound “off” state and a GTP-bound “on” state [51]. The on state of Ras interacts with a number of effectors and activates different pathways that control cell growth or cell death [51]. NO activates Ras by *S*-nitrosylation of Cys-118 and enhances the binding of Ras to GTP [52]. For example, in ischemic preconditioning, it has been shown that activation of p21Ras by NO can promote tolerance and cell survival in the face of future ischemic challenge [6].

17.6 PHYSIOLOGICAL FUNCTION OF *S*-NITROSYLATION

In addition to nitrosylation having specific effects on different pathways at the cellular levels, nitrosylation plays a major regulatory role in the cardiovascular system. For instance, nitroglycerin has been used in the treatment of angina pectoris for more than a century because of the vasodilating action of NO. NO induces vasodilatation by activating GC to convert GTP to cGMP, which can subsequently activate G kinase and phosphorylate proteins that induce blood vessel smooth muscle relaxation [53]. It has been proposed that nitrosothiols carried by hemoglobin play an important role in the regulation of blood circulation in response to metabolic demands [54–56]. One model suggests that NO is carried by oxyhemoglobin in its β -globin Cys-93 residue as nitrosothiol [54–56]. In tissues with low O₂ tension, O₂ will release from the hemoglobin and the release of O₂ will promote the transfer of NO from the cysteine to the heme group [54–56]. This transfer will subsequently allow the NO to be released

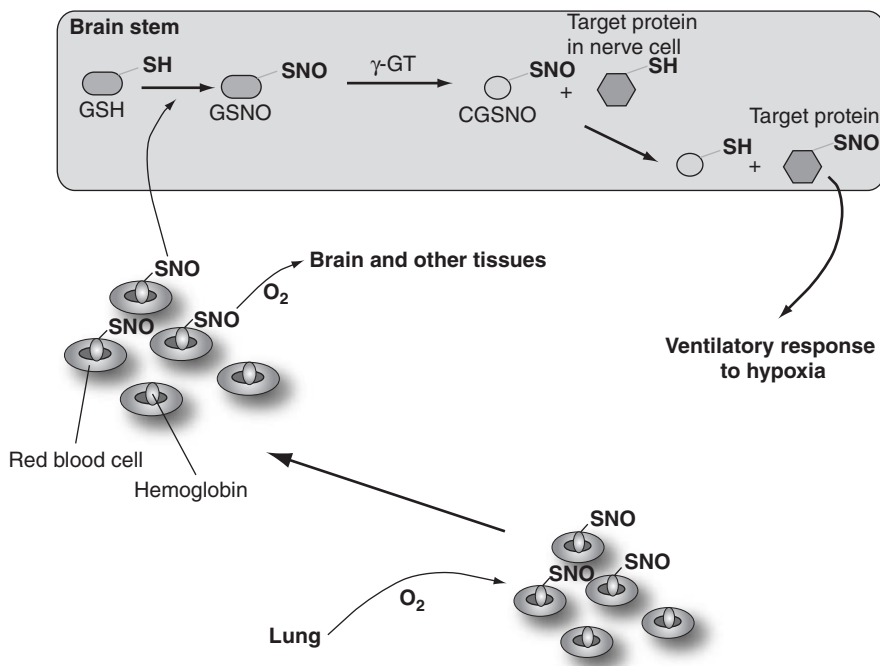


Figure 17.5 Ventilatory response mediated by NO through S nitrosylation. Oxyhemoglobin from the lung releases O_2 in the brain and tissues with high metabolic demands. In the brain stem, low O_2 tension promotes the release of both O_2 and NO. The released NO can transfer to neurons and react with glutathione (GSH) to form GSNO. GSNO can then be metabolized by γ -GT to form CGSNO. The produced CGSNO can then promote the S nitrosylation of targets in the cell and mediate the ventilatory response to hypoxia [57, 58]. (See color insert.)

from the hemoglobin and enhance vasodilation in tissue with low O_2 tension. Therefore, metabolic demands in active tissues can be met by the vasodilation induced by NO [54–56]. This model provides an attractive mechanism of how NO can regulate blood circulation in tissue with high metabolic rate, but critics have questioned the existence of nitrosylated hemoglobin under physiological conditions and argued that this can only occur in experimental conditions when a bolus of NO is applied [55]. Another controversy of this model is that some studies have suggested that NO is being consumed rather than conserved by oxyhemoglobin [55].

NO released from deoxygenated blood can induce hyperventilatory responses during hypoxia through the action of nitrosothiols in the brain stem [57, 58] (Fig. 17.5). Infusions of nitrosothiols such as GSNO, CGSNO, and S-nitroso-L-cysteine (CSNO) into the nucleus tractus solitarius (NTS) of freely moving conscious rats result in a significant increase of minute ventilation which mimics the physiological response to hypoxia [57]. More importantly, nitrosothiols derived from low-mass fractions of deoxygenated blood could induce similar effects as GSNO, CSNO, and CGSNO, and that UV photolysis release of NO from nitrosothiols derived from deoxygenated blood can completely abolish its effects on minute ventilation. Interestingly, when NTS is treated with a γ -GT inhibitor, acivicin, the effect of GSNO-mediated increases in minute ventilation is completely attenuated, which

indicates that the metabolism of GSNO to CGSNO is essential for this physiological effect of nitrosothiols on respiration [57]. These results strongly support the notion that NO plays an important regulatory function in overall respiration and cardiovascular homeostasis.

Because of the importance of nitrosothiols, one would expect that their levels are tightly controlled. GSNOR appears to have high affinity in metabolizing GSNO. Since GSNO is regarded as a major buffering reservoir of nitrosothiols, one would expect that impairment of its metabolism might contribute to a significant disturbance of physiological homeostasis of NO-controlled pathways. In fact, yeast cells deleted of GSNOR show increased susceptibility to nitrosative challenge [16]. In GSNOR knockout mice, they reproduce and develop normally without any morphological or histological abnormality [59]. GSNOR mice also display normal basal blood pressure, and the basal levels of nitrosothiols in blood are normal. However, they display hypotension when anesthetized with urethane [59]. One of the significant differences of these mice is that when they are challenged with endotoxin like lipopolysaccharide (LPS), they display increased levels of protein *S*-nitrosylation, tissue damage, and mortality rate [59]. The enhancement of endotoxin-induced damage can be partially reversed by treating the GSNOR knockout mice with iNOS inhibitor before the LPS treatment [59]. Taken together, these results support the importance of GSNOR when animals are exposed to increased nitrosative stress.

17.7 NITRIC OXIDE AND NEURODEGENERATION

Neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) affect an increasing number of people as the life span of the general population increases. These disorders are usually marked by a progressive loss, associated with aging, of a selective group of neurons. One of the common features of neurodegeneration is the presence of protein aggregates and increased indices of oxidative stress [60–62]. The oxidative stress is particularly viewed as a prime suspect in the pathogenesis of neurodegeneration in relation to aging [60, 61]. For instance, increased lipid peroxidation and nitration of proteins are commonly observed in brain tissue of AD patients [60, 61]. In PD, postmortem pathological studies have revealed that mitochondria complex I dysfunction, reduced glutathione and ferritin levels, and increased lipid peroxidation and iron levels are common [63]. In amyotrophic lateral sclerosis (ALS) patients, consistent increases of 3-nitrotyrosine and nitrated proteins are observed [64]. In all of these disorders, one of the common evaluated oxidative stressors is nitrosative stress. The significant role of nitrosative stress in neurodegeneration can be attributed in part by high NOS activity in the brain and also the intensive energy demand of neurons [65]. The notion that NO is an important player in neurodegeneration is supported by the observation of widespread nitration of pathological inclusions both in neurodegenerative synucleinopathies and tauopathies [66, 67]. It is generally believed that NO mediates its action in the pathogenesis of neurodegeneration mainly through the formation of peroxynitrite, which can damage proteins by reacting with tyrosine residues to form 3-nitrotyrosine, induce peroxidation, and cause DNA damage [68]. However, recent studies have suggested that NO can also contribute to the pathogenesis of PD through *S*-nitrosylation.

17.8 NITROSYLATION AND PD

Parkinson's disease is a common neurodegenerative disorder characterized by movement impairments such as bradykinesia, tremor at rest, rigidity, gait abnormalities, and postural instability [69]. Pathologically, PD is marked by a selective loss of dopaminergic neurons in the substantia nigra par compacta (SNc) and the presence of proteinaceous intracellular inclusions called Lewy bodies [69]. Neuro-biochemically, complex I dysfunction, reduced glutathione, increased lipid peroxidation, and increased levels of iron are also prominent in PD patients due to high level of oxidative stress [69]. The etiology of PD is not known, but it is believed that both environmental and genetic factors contribute significantly to the pathogenesis of PD [69]. This notion is based on the fact that although most PD is sporadic, rare familial cases are also found.

The contribution of environmental factors in PD is confirmed by the discovery of a group of people who developed parkinsonism after the injection of a synthetic heroin in 1980s [70, 71]. Later investigations confirmed the cause of the parkinsonism was due to an impurity, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in the synthetic heroin [70, 71]. The fact that a chemical can cause the development of PD has elicited an intense interest to find out how MPTP induces dopaminergic degeneration. After injection to the vascular system, MPTP crosses the blood–brain barrier, and once in the brain, it is metabolized by monoamine oxidase B (MAOB) to MPP⁺ [72]. MPP⁺ is then taken up by the dopamine transporter (DAT) into dopaminergic neurons and inhibits complex I in the mitochondria [72]. The inhibition of complex I in the mitochondria results in the production of highly reactive superoxide anions and damages different components in the cell. The superoxide anion also reacts with NO to produce the highly toxic peroxynitrite, which can induce lipid peroxidation and protein and DNA damage and ultimately promotes neuronal cell death.

Apart from environmental factors, genetic components are also believed to contribute significantly to the pathogenesis of PD. In fact, 11 loci have been linked to a rare form of familial PD, and mutations in five genes, including *α-synuclein*, *LRRK2* (leucine-rich repeat kinase 2), *PINK1* (PTEN-induced putative kinase 1), *DJ-1*, and *parkin*, cause hereditary PD [73]. Mutations and triplication of *α-synuclein* and mutations in *LRRK2* cause an autosomal dominant form of PD whereas mutations in *PINK1*, *DJ1*, and *parkin* are linked to an early-onset autosomal recessive form of PD (ARPD). The study of these genes is of intense interest; understanding their function might provide insights into the pathogenic mechanism of PD. This notion is supported by the fact that the linkage of *α-synuclein* to familial PD has led to the discovery that *α-synuclein* is also a major component of Lewy bodies.

Nitrosative stress has also long been suspected to contribute significantly to the pathogenesis of PD [74]. For instance, in the MPTP model of PD in rodents, both nNOS and iNOS knockmice are resistant to MPTP toxicity [72]. In postmortem studies of PD patients, increased nitrotyrosine immunoreactivity and nitrated *α-synuclein* in Lewy bodies are prominent in different brain regions [66, 67, 75]. Although nitrosative stress is viewed as an important factor in the development of PD, the role of nitrosylation is also emerging as an important player in the pathogenesis of PD [74]. *Parkin*, a familial PD-linked gene product, was found to

be *S*-nitrosylated by NO both *in vitro* and *in vivo* [23, 76]. Parkin is a RING finger E3 ligase in the ubiquitin pathway, which targets a number of substrates that are believed to be critical for the survival of dopaminergic neurons [77]. Mutations of *parkin* cause an autosomal recessive form of juvenile parkinsonism, which was first identified in a group of PD patients in Japan [63]. It was later found that mutations in *parkin* could account for up to 40% of the early-onset PD cases [78]. Several studies have shown that parkin can function as a multipurpose neuroprotectant, and this function is dependent on its normal E3 ligase activity [77]. Therefore factors that can interfere with parkin's enzymatic activities might affect the survival of dopaminergic neurons in the face of unfavorable conditions [77]. Parkin consists of a "really interesting new gene" (RING) domain, which is conserved in a group of E3 ligases in the ubiquitin pathway [78]. The RING finger domain is characterized by the presence of a cluster of conserved cysteine residues in the motif, which is critical for the function of the protein. The cysteine-rich domains represent a potential target for *S*-nitrosylation to control the enzymatic activity of parkin. Parkin is modified by NO through *S*-nitrosylation, and *S*-nitrosylation of parkin results in a biphasic modulation of its E3 ligase activity [23, 76]. The *S*-nitrosylation of parkin results into an initial increase followed by a decrease in its E3 ligase activity [23, 76]. The consequence of this biphasic modulation of parkin's E3 ligase activity is not clear, but the chronic inhibition of parkin's activity by the high level of nitrosative stress presents a significant burden for the survival of dopaminergic neurons. This notion is supported by the increase in the levels of *S*-nitrosylated parkin in postmortem brain tissues from PD patients [23, 76]. In the MPTP model of PD, mice treated with MPTP or rotenone there is increased *S*-nitrosylation of parkin. Interestingly, MPTP-induced toxicity in mice results in a biphasic increase of parkin *S*-nitrosylation, which is related to the activity of iNOS and nNOS [23]. ESI-MS and biochemical studies have revealed that the sites of modification are located within the first RING finger and in between RING (IBR) domains, which are known to be important for the function of parkin [23, 76]. The finding that parkin can be *S*-nitrosylated and this modification is substantially increased in PD patients provides a common pathogenic pathway for both the sporadic and the familial forms of PD. In sporadic PD, environmental contaminants can result in increased levels of nitrosative stress and hence lead to the inhibition of parkin E3 ligase activity, which is critical for the survival of dopaminergic neurons. The chronic inhibition of parkin E3 ligase activity by constant elevated levels of nitrosative stress might ultimately promote dopaminergic neuronal cell death and contribute to the development of PD.

17.9 CARBON MONOXIDE AS SIGNALING MOLECULE

Carbon monoxide is well known as a toxic gas produced during incomplete combustion of organic fuel. In animals, CO is generated by heme oxygenase (HO), an enzyme that metabolizes heme to CO and biliverdin [79]. There are two isoforms of HO that have been characterized genetically, HO1 and HO2 [79]. HO1 is inducible under various cell stresses and HO2 is constitutively expressed mainly in brain and testes [2]. Studies have suggested that CO produced from inducible HO1 under cellular stressors might represent a signaling molecule for protective mechanisms in cell survival [2]. HO2, on the other hand, is localized specifically in a selected

population of cells such as olfactory neurons and neurons in the enteric system [80]. The signaling mechanism of CO has been attributed mainly to its action on soluble GC. Regulations of soluble GCs in olfactory and intestinal neurons by CO have been reported, but the exact mechanism is not as clear as the activation of GC by NO [80]. NO and CO may act together to mediate signal transduction in blood vessels [2]. For instance, it was found that both NO and CO can act on GC to dilate blood vessels [80]. In contrast, neuronal culture experiments have suggested that CO can antagonize NO-induced increases in cGMP [80]. Therefore the exact mechanism of how NO and CO can cooperate in signal transduction still awaits further elucidation.

17.10 CONCLUDING REMARKS

Nitric oxide is an important signaling molecule that is involved in the control of a number of cellular and physiological processes. The mechanism of how such a gaseous molecule can achieve this function is beginning to be unveiled through recent studies. *S*-nitrosylation seems to be one of the important redox signaling mechanisms of NO. The continuous study of this pathway will definitely help to improve our understanding of pathogenic mechanisms of different disorders in humans and should provide critical information for the development of new pharmaceutical targets in the near future.

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18

NEUROBIOLOGY AND TREATMENT OF DEPRESSION

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18.1	Introduction	765
18.1.1	Current Therapeutics Issues	766
18.1.2	Adequacy of Current Antidepressants	766
18.2	Neurochemistry of Mood Disorders: Monoamines and Their Signal Transduction Systems	766
18.2.1	Noradrenergic System	767
18.2.2	Serotonin System: New Understanding of New Drugs	769
18.2.2.1	The 5-HT _{1A} Receptor	770
18.2.2.2	DARPP-32 and Mechanism of Action of SSRIs	771
18.2.3	Monoamine Transporters	772
18.2.4	Regulators of G-Protein Signaling as Potential Drug Targets in Mood Disorder	772
18.2.5	Neuroplasticity and Use-Dependent Activity	773
18.2.6	Neurotrophic Hypothesis in Depression	773
18.2.7	BDNF Clinical Findings	775
18.2.8	Potential Pharmacophores to Modulate Neurotrophin Expression	776
18.2.9	Excitatory Amino Acid Receptors and Transporters in Neurons and Glia	777
18.2.10	Clinical Evidence for NMDA Receptors in Mood Disorders	778
18.3	Conclusions	779
	References	780

18.1 INTRODUCTION

Depression is one of the most costly brain diseases in the world in terms of pain and suffering, lost work, and mortality from suicide. Depression is not only common but also appears to be increasing in frequency and to be occurring at an earlier age of

onset. Disorders of mood are increasingly understood to be a group of systemic illnesses involving central neurotransmitter imbalances and neuroanatomical disruptions, along with potential dysregulation of immune, autonomic, endocrine, and cardiovascular function. In this chapter emphasis is placed on recent advances in depression research and a discussion is presented of future directions that might catalyze discovery of innovative treatments.

18.1.1 Current Therapeutics Issues

Depression is underdiagnosed and undertreated, even after observed suicidal behavior has been noted [1]. It is associated with lifetime suicidal behavior in 10% of cases, with approximately 3% completing suicide, predominantly men [2]. The one-month U.S. prevalence for depression ranges between 1.5 and 2.5% and the lifetime prevalence is approximately 17% [3]. Employing the disability-adjusted life years (DALYs) methodology, the Global Burden of Disease Study calculated that depression is presently ranked fourth but by 2020 will become the second leading cause of disability, trailing only heart disease (and followed by traffic accidents) [4]. Already in the 15–44-year age group, depression is the leading cause of DALYs worldwide.

18.1.2 Adequacy of Current Antidepressants

Residual symptoms after treatment are more the rule than the exception, and there is concern that further research will conclude that chronicity leads to progressive treatment resistance. This has led to a shift in zeitgeist with new emphasis on treating both acute and residual symptoms more aggressively, with a close eye being kept on functional impairment [5].

About 30% of patients with depression do not meet typical response criteria to an initial course of antidepressants, and furthermore the majority of patients are not symptom free with monotherapy. Additionally, 10–20% of depressed patients do not tolerate an initial trial of antidepressant medication. Thus, 25–35% of patients who complete an adequate trial of an approved antidepressant do not have an adequate response [6]. Moreover a recent clinical meta-analysis indicates that serotonin selective reuptake inhibitors (SSRIs) and the dual monoamine reuptake inhibitor venlafaxine achieve *remission*, as defined, in only 35 and 45% of patients, respectively [7].

These studies highlight the importance of defining novel targets for treatment of people with depression, an area of intensive research effort around the world.

18.2 NEUROCHEMISTRY OF MOOD DISORDERS: MONOAMINES AND THEIR SIGNAL TRANSDUCTION SYSTEMS

There is a broadly acknowledged scarcity of validated drug targets in clinical discovery in psychopharmacology, related in part to the lack of understanding of pathophysiology, poorly differentiated clinical targets (i.e., objective diagnoses), and an absence of objective tests. It is now clear based on various lines of reasoning and empirical data that changing the set point of monoamine transmission does not fully explain antidepressant action but plastic changes in the limbic target areas of

monoamine neuron projections are important in the mechanism of action of antidepressants.

Antidepressants affect multiple presynaptic and postsynaptic processes: for example, changes in cyclic adenosine monophosphate (cAMP) signaling, altered protein kinase or phosphatase activity, changes in neuroplasticity via alteration of neurotrophin expression and neurogenesis, alteration of presynaptic function through effects on synaptic vesicle proteins, changes in activity or receptor binding at specific monoamine receptor subtypes (e.g., α_{1a} receptors), alterations in neuropeptide neurotransmitter binding, upregulation of stress-related receptors such as the glucocorticoid receptors, acting as antagonists or agonists at various steroid receptors, acting as corticotropin-releasing hormone (CRH) receptor antagonists or via alteration of levels of CRH, antagonizing vasopressin receptors, changes in ion channel function including alteration of ion flux and concentrations such as calcium, and alterations in glutamate and γ -aminobutyric acid (GABA) neurotransmission. Further adding to the complexity is new evidence that monoamine neurotransmitters not only are released at terminal fields but also can be released by the *soma and dendrites of neurons themselves* [8]. Thus, antidepressants may effect nonclassical neurotransmitter release. The “conceptual wilderness” in our understanding of antidepressant action since the discovery of the monoamine-based antidepressants [9] will hopefully soon give way to a rich understanding of how interventions in multiple systems and at multiple system levels are therapeutic in subpopulations.

It appears likely that in major depression antidepressants ultimately alter homeostasis or allostasis through complex signaling pathways that affect transcriptional events, the activity of enzymes at the cellular membrane, and patterns of neuronal system activation and connectivity. Key drug targets are critical and possibly unique “biochemical switches” that can be adjusted to alter mood physiology for therapeutic effect, and biologically defined subtypes are needed to further progress in therapeutic development. It appears that the behavioral effects of norepinephrine (NE), serotonin, and dopamine have considerable overlap such that augmenting levels of any one may have antidepressant effects and increasing synaptic levels of more than a single neurotransmitter may be synergistic [7]. Crosstalk between NE neurons, dopamine, and 5-hydroxytryptamine (5-HT) neurons has been documented such that increased NE stimulates 5-HT and dopamine release and 5-HT release at NE neurons reduces NE release. As another example, blockade of the *norepinephrine* reuptake receptor can reduce the uptake of *dopamine* in the frontal cortex since the norepinephrine transporter (NET) has high affinity for dopamine (in fact NET has a higher affinity for dopamine than does the dopamine transporter itself), and dopamine transporters in any event are found at low levels in frontal cortex, suggesting that drugs that inhibit the NET may be antidepressant by affecting prefrontal dopamine signaling [10]. Further study of patterns of reciprocal interaction and crosstalk between these nondiscrete systems may allow us in the future to understand the balance of activity of monoamine systems and tailor therapy [11–14].

18.2.1 Noradrenergic System

The locus ceruleus (LC) is a compact nucleus containing noradrenergic neurons as well as peptide neurotransmitters (e.g., hyopretin and CRH) that influence its activity (for a recent review see [15]). LC neurons can fire in either a tonic or phasic

pattern and electrotonic coupling between neurons can be influenced by neurotransmitters. Release of NE can be accompanied by corelease of the peptide neurotransmitter galanin, which is inhibitory and may alter the firing rate of dopamine neurons, thus altering its hedonic tone. Shifts in the pattern of firing of LC neurons are thought to be of great importance in understanding attentional processes, often disrupted in depression. The LC neurons have long dendritic processes for synaptic contact to influence its activity, and LC neurons may be strongly influenced by anterior cingulate cortex. It has recently been suggested that the A2 group of the medulla may innervate important structures such as the amygdala and nucleus accumbens and thus may be important in affect regulation. Receptors for NE are grouped into α_1 , α_2 , β_1 , and β_2 subtypes.

Chronic therapy with antidepressants results in adaptive receptor alterations in the noradrenergic system. Three genes code for the expression of α_1 subtypes, and these three receptor subtypes (A–C) have distinctive pharmacological properties. Recent data suggest that chronic antidepressants and electroconvulsive stimulation (ECS) may increase frontal cortex expression of messenger ribonucleic acid (mRNA) *specifically* for the α_{1A} adrenoreceptor subtype and as such this receptor may be involved in the action of noradrenergic antidepressants [16]. Repeated administration of antidepressants has been observed to increase behavioral responsivity to α_1 adrenergic agonists (such as aggressiveness and hyperexploration) as well as increasing agonist binding affinity for α_1 adrenoreceptors.

Electrophysiological studies in the hippocampus also support enhanced α_1 responses after chronic antidepressant treatments. Recent data indicate that the novel antidepressant tianeptine, which may increase serotonin reuptake when given chronically, also increases responsiveness of the α_1 -adrenergic system [17].

Single-unit recording from the LC indicates that chronic administration of multiple classes of antidepressants and electroconvulsive stimulation reduce LC baseline and sensory-stimulated firing rates [18]. It has been hypothesized that reducing the firing rate of noradrenergic neurons may be therapeutic, especially in depression with certain clinical features such as psychomotor retardation by reducing the release of inhibitory coreleased galanin neuropeptide onto dopamine neurons in the ventral tegmental area (VTA) [19]. However, reduced firing in the noradrenergic neurons of the LC could simply be a function of increased levels of synaptic or extracellular NE resulting in feedback inhibition of LC firing [18].

The α_2 antagonist yohimbine has been observed to augment the speed of response to fluoxetine [20], and in a very small study ($n = 14$) of bipolar depression the α_2 antagonist idazoxan appeared to have antidepressant effects equal to bupropion [21]. Upregulation of immunolabeled α_{2A} receptors and associated G proteins (G_i) are observed postmortem in suicide victims [22]. This is of interest given the critical importance of these receptors in stress and in regulating levels of monoamines via autoreceptors and heteroreceptors. As well, the efficacy of antidepressants such as mirtazapine and mianserin may in part depend on these receptors. Recent transgenic experiments suggest that the α_2 receptor may act as a “suppressor of depression.” Knockout of the gene for the α_2 receptor increases immobility in the forced-swim test and eliminates the augmentation of forced-swim test activity by imipramine [23]. In contrast, other recent experiments suggest that mice lacking α_{2c} receptors perform on the forced-swim test in the *same* fashion as mice *treated* with antidepressants [24]. Thus, the α_{2a} and the α_{2c} receptors may have complementary and *opposing* roles in

the regulation of mood [25]. If reducing α_{2c} activity is to be used as an antidepressant strategy, it may require some method of targeting only those receptors in the central nervous system (CNS) since an α_{2c} (Del322–325) polymorphism that reduces feedback inhibition of sympathetic NE released in the heart is associated with a markedly increased risk of heart disease [25]. Since some individuals with depression also have memory disturbance, recent evidence that mutation of the α_{2a} receptor impairs working memory could also help us understand the cognitive symptoms observed in depression [26].

Crosstalk between the catecholamine system and steroids may be another novel mechanism through which NE and epinephrine by increasing the sensitivity of glucocorticoid receptors (GRs) to ligand activation could alter mood. A recent study found that amitriptyline prevented the appearance of impairment in spatial memory in aged rats and reduced glucocorticoid levels, and this effect is most likely secondary to NE-mediated alteration in glucocorticoid signaling [27]. Augmentation effects of catecholamines on GR signaling may thus be important in cognitive and emotional processing. The phosphatidylinositol-3 kinase (PI₃-K) signaling pathway activation through β receptors appears to be responsible for this putative enhancement of glucocorticoid activity, and it is tempting to conjecture that antidepressants which are known to downregulate β receptors and influence PI₃-K signaling could act by glucocorticoid receptor sensitization.

Clinical evidence also suggests noradrenergic system involvement in severe depression. Levels of the rate-limiting enzyme for catecholamine synthesis, tyrosine hydroxylase (TH), are upregulated postmortem in suicide victims [28], and chronic treatment with all of classes of antidepressants reduces activity of TH in rodents [29]. In recent years experiments with the catecholamine-depleting agent α -methyl-*p*-tyrosine (AMPT) by Miller and colleagues suggests that some patients with depression, posttreatment, are dependent on specific monoamines to maintain normal mood. Thus, depletion of NE via AMPT challenge in subjects that responded to a predominantly noradrenergic antidepressant but not those responsive to and SSRI results in brief relapse [30]. In addition it appears that treatment refractory depression may be more responsive to agents that augment NE in addition to serotonin as evidenced by robust response rates with venlafaxine and duloxetine and with mirtazapine augmentation studies [7, 31, 32].

18.2.2 Serotonin System: New Understanding of New Drugs

The indoleamine serotonin has a major role in the treatment of various mental disorders and the SSRIs are the most frequently prescribed class of drugs to treat depression in the United States. However, the biochemical mechanism through which serotonergic fibers interact is complex. Neurons, glia, and endothelial cells possess at least 14 distinct receptors, and serotonin is involved “in more behaviors, physiological mechanisms, and disease processes than any other brain neurotransmitter” (reviewed in [33], p. 413). Agents that enhance serotonergic activity such as the SSRIs, which block the serotonin transporter, are effective antidepressants following a substantial latency period. Serotonin in development and adulthood has an important role in CNS neuroplasticity. Clinical and preclinical studies have thus far mostly implicated stimulation and interaction of 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} or

5HT_{2C} receptors in antidepressant action, but this emphasis may in part be an artifact related to the availability of selective ligands for these receptor subtypes.

18.2.2.1 The 5-HT_{1A} Receptor. The 5-HT_{1A} receptor is a seven-transmembrane G-protein-coupled receptor (GPCR) found at both presynaptic locations in the raphe nucleus and postsynaptic locations and is critically involved in regulating mood and anxiety levels. Postsynaptic stimulation in the hippocampus augments synaptogenesis in adult animals via a trophic factor referred to as S-100 β . 5-HT_{1A} receptors also appear to stimulate neurogenesis. 5HT_{1A} receptors signal via a G_{ai}-coupled inhibition of adenylyl cyclase and by hyperpolarization via the opening of a K⁺ channel. 5-HT_{1A} receptor somatodendritic desensitization is considered a key mechanism that allows SSRIs to produce clinical effects in depression by reducing feedback restraint upon serotonin release (reviewed in [34]). However, 5-HT_{1A} autoreceptor downregulation does not appear to be a universal mechanism since tricyclics such as amitriptyline do not cause somatodendritic desensitization [35]. It is also known that a cyclooxygenase-dependent metabolite of arachidonic acid (AA), produced via activation of phospholipase A2 (PLA2), targets the 5-HT_{1A} effector adenylyl cyclase and reduces the responsiveness of the 5-HT_{1A} receptor. This desensitization mechanism is also involved in regulation of the 5-HT_{1B} receptor. 5-HT_{1A} receptor activity is also reduced by activation of protein kinase C (PKC) (via receptor phosphorylation). Neuronal firing rates of serotonin neurons are also affected by feedback loops from the cortex and amygdala. Furthermore, the SSRI fluoxetine reduces hypothalamic sensitivity of 5-HT_{1A} receptors possibly by reducing levels of the G_i family G protein, G_z. Thus, the net effect of antidepressants on 5-HT_{1A} receptors is the result of integration of phospholipid signaling effects on positive and negative regulators, changes in G-protein coupling, and alteration of terminal field feedback [36].

The 5HT_{1A} receptor may counteract the effects of activation of the 5HT_{2A} receptor. Activation of the 5HT_{1A} receptor exerts a hyperpolarizing effect on cortical neurons whereas activation of the 5-HT_{2A} receptor is depolarizing. Activation of 5HT_{2A} receptors results in glutamate release from thalamocortical afferents, and increased levels of glutamate as discussed below may be involved in the etiology of depression. It has been suggested by Marek that either antagonism of 5HT_{2A} receptors or increased activity of cortical 5HT_{1A} receptors would have similar effects of reducing glutamate release (G. Marek, personal communication, 2002). It is tempting to speculate that a drug designed to combine 5-HT_{1A} agonism with postsynaptic 5-HT_{2A} antagonism would have robust antidepressant action.

Recent knockout experiments of the 5-HT_{1A} receptor indicate that the receptor is important early in development with respect to affect-regulated behaviors. 5HT_{1A} null mice have increased anxiety, but “rescue” at a later age in conditional knockouts does not reduce anxiety if the receptor was absent at a developmentally crucial early period [37]. Knockout of the 5HT_{1A} receptor, possibly by eliminating feedback inhibitor, has the effect of reducing immobility in the tail suspension test, simulating antidepressant action. However, rather than being the result of simply increasing synaptic serotonin, challenge studies employing alpha-methyl-para-tyrosine (AMPT) have implicated augmentation of *catecholamine* function in the antidepressant-like behavioral effects of 5-HT_{1A} receptor deletions. However, knockout of the 5HT_{1A} receptor eliminates

the behavioral response to an SSRI, implicating the 5-HT_{1A} receptor directly in their SSRI action [38].

Vilazodone (SB659746-A) is a combined serotonin reuptake inhibitor and 5HT_{1A} partial agonist that provides proof of principle that reduction of somatodendritic 5HT_{1A} receptor activity might provide improved antidepressant effect [39]. Vilazodone augments synaptic serotonin levels compared to conventional antidepressants in rodents and has classic antidepressant effects in the forced swim test (FST). However, there is an inverted-U-shaped curve such that at high doses it loses its antidepressant-like behavioral effects. This nonlinear behavior of the drug may indicate that at low doses, due to vilazodone's partial 5HT_{1A} agonist effect, it relatively reduces somatodendritic activity or facilitates desensitization of these receptors while at higher doses it begins to antagonize activity at postsynaptic limbic 5HT_{1A} receptors, which diminishes its antidepressant effect.

Paradoxical [rapid eye movement (REM)] sleep is increased in stress and in depression, and depressed subjects may have a decreased number of postsynaptic 5-HT_{1A} receptors. Like depressed humans, 5-HT_{1A} knockout mice have abnormally increased density of REM sleep and do not have normal REM sleep rebound after stress or sleep deprivation [40]. Since 5-HT_{1A} receptors and 5-HT_{2A} receptors have antagonistic effects on intracellular signaling, increased sleep continuity with 5-HT_{2A} antagonists might be related to a relative increase in 5-HT_{1A} signaling.

18.2.2.2 DARPP-32 and Mechanism of Action of SSRIs. Attempts at understanding the temporal delay in onset of antidepressant effects have recently focused on signal transduction cascades and changes in gene expression. Evidence has suggested that antidepressants, including the SSRIs, augment activity in the cAMP-dependent protein kinase A (PKA) pathway in the cortex and hippocampus [41]. DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of molecular weight 32,000) is a protein concentrated in the prefrontal cortex and striatum. Chronic treatment of mice with fluoxetine increases levels of DARPP-32 in the prefrontal cortex and hippocampus. DARPP-32 regulates the efficacy of various ion channels and ionic receptors via phosphorylation. Serotonin acts on DARPP-32 synergistically through 5-HT₂, 5-HT₄, and 5-HT₆ receptors to convert DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP-1), which results in reduced protein dephosphorylation. As a result, transcription factors that may be relevant in the treatment of depression and in neurodegenerative disorders such as cAMP response element binding protein (CREB) would have increased activity. Other antidepressants and lithium also increase levels of rat frontal cortex DARPP-32. Since antidepressants may act through the brain-derived neurotrophic factor (BDNF), it is interesting to note that BDNF KO mice have significantly reduced levels of DARPP-32. A receptor regulated by DARPP-32 is the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, a glutamate receptor subtype implicated in depression and cognition, as discussed below. Chronic treatment with fluoxetine increases phosphorylation of the GluR1 AMPA receptor subunit and the DARPP-32/PP1 signaling cascade is involved in this process. In DARPP-32 KO mice fluoxetine is significantly less efficient in augmenting phosphorylation of AMPA GluR1 subunits. As well, DARPP-32 KO mice have a markedly reduced response to fluoxetine in the tail suspension test for antidepressant efficacy. Therefore a novel mechanism of action for SSRIs may be via alteration of "kinome"-regulated processes (i.e., changes in

kinase/phosphatase balance) that increase AMPA receptor currents and result in AMPA receptor-regulated changes in synaptic plasticity [42, 43].

18.2.3 Monoamine Transporters

Serotonin transporters (SERTs) and NETs are both high-affinity targets of antidepressant agents and both transporters are the product of a single gene residing in the same gene family. Blakely has noted that a single gene coding for transporters indicates that polymorphisms that affect the function of the receptor and thus the clinical phenotype will be expressed in both the CNS and periphery transporters [44]. Transporter expression and sequestration are regulated by kinase activity such as PKC, and SSRIs alter the gene expression of the kinase involved in the regulation of SERT expression.

As mentioned, NET inhibitors (e.g., desmethylimipramine, which is a highly selective NET inhibitor) have been used as antidepressants for many years. Recently, the additional two highly selective NE reuptake inhibitors reboxetine and atomoxetine were marketed. Recent transgenic experiments support potential importance for NET in depression. Xu and colleagues observed that NET knockout mice behaved as if treated with antidepressants in that they had locomotor hyperresponsiveness to stimulants [45]. However, reduced levels of NET have been observed in the LC postmortem in depression, possibly reflecting an adaptive response to decreased levels of extracellular NE [46].

NET polymorphisms have been identified in association with abnormal cardiovascular function. A proof-of-concept study identified polymorphism is based on observation of patients with orthostatic intolerance [47] along with fatigue, headaches, syncope, and attention-deficit symptoms [44]. Peripheral manifestations of NE or serotonin system abnormalities could be important markers for identifying and subtyping mood and anxiety disorders or enriching clinical samples for genetic studies or pharmacological trials.

18.2.4 Regulators of G-Protein Signaling as Potential Drug Targets in Mood Disorder

The dramatic efficacy of antidepressants effecting biogenic amines is related to their powerful influence on the family of GPCRs. The biogenic amine antidepressants alter receptor thermodynamics toward a distinct drug-bound ensemble. GPCR activation alters the specific intracellular ensemble of G proteins and may result in receptor internalization or phosphorylation and increases interaction with auxiliary membrane affiliated proteins. Antidepressants thereby alter the activity of enzymes in multiple signal transduction pathways that modify neurophysiological response [48].

GPCR signal transduction has been considered as a three-component model with the seven-transmembrane surface receptor coupled to a membrane-associated heterotrimeric G-protein complex. Recent data indicate that GPCRs form homo- and hetero-oligomeric complexes, and this revelation may prove important for future drug design and for understanding complex neurotransmitter system interactions and synergisms. Binding of guanosine triphosphate (GTP) to a G α switch region engages a specific effector for each G α subtype [49].

A newly discovered component of the GPCR signaling cascade is a family of proteins known as regulators of G-protein signaling (RGS proteins). RGS proteins are mainly GTPase-accelerating proteins (GAPs) for $G\alpha$ (i.e., they augment G-protein inactivation), and thus potential small-molecule *inhibitors* of tissue-specific RGS proteins should lead to enhanced signaling from agonist-bound GPCRs. Wyeth-Ayerst Research has described transgenic rodents that are insensitive to RGSs at specific $G\alpha$ subtypes in the CNS. They exhibit heightened serotonin receptor signaling at 5-HT₂ receptors and muscarinic receptors. The prolonged signaling observed via $G_q\alpha$ -PLC β is similar to that seen after lithium administration in normal rodents. Electroconvulsive seizures are known to increase CNS expression of RGS2 in the amygdala, cortex, striatum, and hippocampus, while RGS10 is decreased in dentate gyrus and parietal cortex. Unpredictable stress or corticosterone increases LC and hypothalamic expression of RGS4. RGS7 is increased in brain of mice treated with the proinflammatory cytokine tumor necrosis factor- α (TNF- α).

Tissue-specific enhancement of GPCR signaling by RGS small-molecule inhibitors is thus a novel antidepressant strategy and could greatly increase signal throughput via D2/D4 receptors in the basal ganglia and a RGS9 inhibitor plus a D2 agonist may be effective at lower doses and with fewer side effects in Parkinson's disease and depression. Thus, novel therapeutics through either stimulation or inhibition of RGS proteins in specific brain regions may enhance catecholamine or serotonin signaling or have mood-stabilizing effects similar to lithium.

18.2.5 Neuroplasticity and Use-Dependent Activity

Preliminary insight into the mechanism underlying an organism's ability to adapt to and learn from its environment began with the observation that neurotransmitter stimulation of neurons alters gene transcription. Use-dependent plasticity is seen in neuronal networks and synapses, and the downstream expression of transcription factors that sculpt neural circuits is critical to our understanding of mood states and antidepressant action. The term "neuroplasticity" subsumes *functional changes* in the nervous system and can be discussed at different levels. Plasticity can be at the level of synaptic anatomy and connectivity—changes in synaptic plasticity via alterations of dendritic function, synaptic remodeling, synaptogenesis, or neurogenesis. Plasticity can also refer to changes in long-term potentiation or be discussed in terms of behavioral plasticity [50]. Neurotransmitters, neuromodulators, and hormones that influence intracellular signaling cascades mediate these functional changes. Recent morphometric studies and pharmacological studies suggest the possible importance of neuroplastic events in the etiology and treatment for affective disorders [51–54].

18.2.6 Neurotrophic Hypothesis in Depression

The neurotrophins are a family of related, secreted peptides that powerfully affect development and the adult functioning or plasticity of the CNS [55] (see also chapter 8 in volume III of this handbook). Neurotrophins regulate neuronal survival and control neurite growth in either a positive or negative direction via interactions or functional interplay between two types of transmembrane glycoprotein receptors. Thus neurotrophins are critical for *selection of functional neuronal connections in the CNS*, which makes them prime candidates for understanding the complex neurodevelopmental and

environmental factors in depression. Neurotrophins signal via the *trk* family of receptor tyrosine kinases, which have specificity for neurotrophin ligands, and via the pan-neurotrophin receptor, p75 (p75^{NTR}), which is a common receptor for the whole neurotrophin family of ligands. BDNF infusion after neurotoxin exposure markedly enhances plasticity and neurite outgrowth in the serotonin system of the adult rodent brain [56] (see also Chapter 20). Intermediate doses of BDNF were most effective, suggesting an inverted-U-shaped dose–response curve.

In recent years Duman and the Yale group have proposed a new dramatic molecular and cellular theory of depression [57, 58] (see also Chapter 20) based on increased knowledge of the intracellular cascades that are stimulated by antidepressants that result in the generation of BDNF, which along with NT 4/5 activates TrkB receptors. TrkB receptors are located both in GABAergic interneurons and glutamatergic pyramidal neurons and granule cells. TrkB receptors are also found in the raphe neurons of the rat raphe nucleus [59]. BDNF has both presynaptic effects that facilitate neurotransmitter release and postsynaptic effects [60, 61] on plasticity. BDNF affects the balance of potentiation and inhibition in circuits by augmentation of the level of AMPA receptor–mediated miniature excitatory postsynaptic currents (mEPSCs) and by increasing the frequency of miniature inhibitory postsynaptic currents (mIPSCs) [62].

The capacity of neurotrophic factors to provide trophic support and influence neuroplasticity may be through activation of the mitogen-activated protein kinase (MAPK) pathway, which includes the extracellular signal-regulated kinase (ERK), and via the PI₃-K/Akt signaling pathway. If neurotrophic factors are involved in depression, it could indicate a primary deficiency of neurotrophic factors or that increased levels of such factors are necessary to overcome reduced functioning of the MAPK/ERK signaling pathways critical for transactivation of specific genes. Protection against excitotoxicity and neuroendangerment and increased levels of BDNF may result from induction of c-Fos that heterodimerizes with c-Jun to form the AP-1 transcription factor. Thus, mice in which c-Fos has been knocked out have increased excitotoxicity and neuronal cell death and altered levels of BDNF [63].

A critical transcription family for neurotrophin expression is CREB, which is made up of CREB, CREM (CRE-modulatory protein), and ATF1 (activator transcription factor 1). CREB turns on the BDNF gene (and BDNF release in turn stimulates CREB) and the NMDA receptor–linked molecule nNOS (neuronal nitric oxide synthetase) as examples. Following synaptic activity calcium entry through specific routes stimulates MAPK-dependent phosphorylation of CREB. Calcium can also eventually result in dephosphorylation of CREB via activation of protein phosphatases PP1 and PP2A. The neurotrophic hypothesis of antidepressant action suggests that many antidepressants increase expression of CREB and phosphorylated CREB, which upregulates among its gene targets BDNF [64]. Antidepressants may therefore act by increasing synaptic plasticity in mood disorders [65].

Stress markedly reduces hippocampal neurotrophin levels in model systems [66], and the hippocampal atrophic changes caused by stress are reversible by central BDNF administration in animal models of depression [67]. Stress-related changes in BDNF levels in the hippocampus may result from alteration in the balance of excitatory and inhibitory input as well as circadian and stress-related changes in glucocorticoid levels [68, 69]. Moreover, corticosterone shifts the intracellular location of BDNF away from the nucleus of neurons with concomitant increased cytoplasmic

levels, although the significance of this is unclear [70]. Depressive symptoms including motor slowing and agitation are associated with a decrease in BDNF expression in an ethanol withdrawal model, and fluoxetine both reverses the anxiety phenotype and prevents the decrease in BDNF immunolabeling [71]. However, stress does not universally decrease BDNF expression. In fact, immobilization stress has the *opposite* effect—and thus enhances BDNF mRNA levels and protein in the rat hypothalamus [72], suggesting that BDNF may also be important in the plasticity processes that integrate stress responsivity in the hypothalamic–pituitary–adrenal (HPA) axis.

Antidepressants, but not other psychotropics such as cocaine, increase BDNF and are neuroprotective in animal models [73, 74]. Furthermore, BDNF directly administered into the hippocampus has sustained antidepressant effects in a rodent model of depression as early as three days after a single infusion and the effect size is comparable to that observed after antidepressant administration [75]. Moreover, mice with knocked-down levels of BDNF have abnormal levels of aggression and reduced levels of brain serotonin [76]. Normal levels of BDNF and TrkB signaling are necessary for behavioral response to antidepressants on the forced-swim test. Furthermore, antidepressants are observed to specifically increase TrkB autophosphorylation in the prefrontal cortex, cingulate cortex, and hippocampus, suggesting increased neurotrophic signaling as the mode of action of antidepressant [77].

Further evidence of the critical importance of BDNF for neuroplastic responses to pharmacological stimulation comes from the field of addiction research, where the neuroadaptive effects of opiates on the LC and noradrenergic system are markedly reduced in mice with a conditional knockout of BDNF in the postnatal period [78]. As noted, sleep deprivation can result in a limited antidepressant effect and can induce mania in bipolar patients. In rats it appears that BDNF and TrkB as well as CREB may be upregulated during waking hours and during sleep deprivation [75, 79].

18.2.7 BDNF Clinical Findings

Reductions in BDNF are suspected in neurodegenerative disorders such as Alzheimer's disease [80] and may be reduced in some individuals with depression. In a postmortem study, prefrontal cortex levels of the transcription factor CREB are reduced in major depression and restored in those treated with antidepressants [81], and hippocampal BDNF immunoreactivity is increased in depressed subjects treated with antidepressants. BDNF is increased in the hippocampus in depressed patients who were being treated with antidepressants at the time of death compared to those subjects untreated [82].

BDNF may be transported in a bidirectional fashion across the blood–brain barrier [83]. Positive correlation has been reported between serum and cortical BDNF levels [84]. Karege and colleagues observed in mostly depressed subjects ($n = 30$) that BDNF serum levels were reduced compared to controls, with the greatest decrease in female subjects. There was considerable overlap with controls, but the reduced levels correlated with depression severity in patients [84]. Reductions in serum levels have also been reported in other psychiatric populations, including schizophrenia [84]. It is possible that in a subpopulation, of depressed patients peripheral levels of BDNF or other neurotrophic substances that can be transported to and from the CNS such as insulin-like growth factor-1 (IGF-1) may be candidate biomarkers of disease [85]. Although it is clear that platelets contain significant quantities of BDNF that is

released at sites of injury containing TrkB, the relevance of peripheral BDNF measurements for understanding brain function is unknown [86].

The BDNF gene is located on the short arm of chromosome 11 (11p13–14). There are two studies with suggestive scores of logarithm of the odds of linkage (LOD) to this region of 1.89–1.95 in bipolar pedigrees [87, 88]. BDNF has been suggested as a candidate gene or risk locus for genetic association study [89] and a recent family-based genetic association study has evidence for linkage disequilibrium between specific BDNF polymorphisms and bipolar disorder using the family-based association test. Thus a gene variant in the vicinity of the BDNF gene may confer risk of bipolar disorder [90], though the gene variant's functional significance is unknown. In addition a transmission disequilibrium test found that a specific BDNF haplotype had decreased transmission to bipolar probands, which could suggest that this haplotype is a protective factor against disease [90].

18.2.8 Potential Pharmacophores to Modulate Neurotrophin Expression

Even if the neurotrophic hypothesis of depression is proven to be substantially correct, there is currently an absence of small-molecule agonists for Trk receptors. An alternative strategy is to discover drugs that upregulate Trk receptors or increase their sensitivity. Furthermore, BDNF saturable blood–brain barrier penetration and the existence of truncated TrkB receptors for BDNF that can sponge up growth factors limit our current capacity to augment trophic activity [91]. Moreover, it is now well established that neurotrophins, in addition to involvement in maintaining neurons, have a critical role in moment-to-moment modulation of activity-dependent neuronal plasticity. Hence, indiscriminate or wholesale application of neurotrophins is likely to result in dramatic levels of serious side effects and neuronal circuit dysfunction. It is possible that strategies able to locally supply neurotrophins to projection nuclei such the LC or dorsal raphe may prove successful. Trials thus far using neurotrophin administration for amyotrophic lateral sclerosis and other neurodegenerative disorders have not been unsuccessful [92].

Small-molecule drugs have been found that possess neurotrophic activity. The immunosuppressant FK506 that binds to the immunophilin 12-kDa FK506 binding protein FKBP 12 has neurotrophic properties. Immunophilins were initially identified as receptors for immunosuppressant agents, including cyclosporin A and FK506. Immunophilins are highly concentrated in the brain compared with peripheral tissues. Derivatives of the immunosuppressant drugs have been developed that are neurotrophic without suppressing immunity. Small molecules have been developed such as GPI-1046 with *in vivo* and *in vitro* neurotrophic properties, and these drugs promote, for example, the regrowth of dopamine and serotonin neurons in lesion experiments and may be neuroprotective. These drugs are currently lead compounds for the treatment of neurodegenerative disorders and may be worthwhile testing in severe mood disorder as evidence builds supporting a neurotrophic factor deficiency hypothesis in depression.

Other approaches might be to indirectly stimulate BDNF production by, for example, the use of small molecules that modulate AMPA receptor function known as AMPAkinases (*vide infra*) and BDNF reciprocally modulates the expression of AMPA receptor proteins on neurons [93]. Another mechanism that may be exploited in the future to finely tune the level of neurotrophic activity and neuroplasticity is

changing levels of cyclic guanosine monophosphate (cGMP) or nitric oxide (NO). Recent data indicate that within the hippocampus the NO/cGMP/protein kinase G (PKG) signaling pathway may be critically involved in the rapid downregulation of BDNF [94]. The success influencing this pathway in a tissue-specific fashion in the treatment of erectile dysfunction using sildenafil [95] suggests that CNS-specific drugs may be of interest in treating depression or neurodegenerative disorders. BDNF and neurotrophin (NT) 4/5 via an autoregulatory pathway stimulate further production of neurotrophic factors. These neurotrophic factors may in turn increase their own levels by increasing levels of AMPA ionic glutamate receptor subunit GluR1 [96].

However, there is some evidence that the acute or subacute effects of antidepressants in behavioral immobility antidepressant assays (i.e., the forced-swim test or tail suspension test) are not mediated via CREB-dependent transcriptional products [97]. This was concluded based on experiments with CREB-deficient mice where the behavioral (i.e., decreased immobility in the FST) and endocrine effects of subchronic antidepressants were similar in wild-type and CREB-deficient mice [97]. An additional criticism of the neurotrophic hypothesis is that conditional knockouts for BDNF have an anxious phenotype but do not show obvious signs of depression [98]. As well, in contrast to the hippocampus, it appears that increased levels of CREB and BDNF in the dopamine-rich nucleus accumbens may *reduce* hedonic tone and have a depressogenic effect. It appears that regional overexpression of CREB specifically in the shell of the nucleus accumbens “gates” strong affective experiences with either a positive or negative valence. This is probably mediated by downstream influences on the regulation of peptide or nonpeptide neurotransmitters or neuromodulators controlled by CREB sites [99]; thus CREB in the nucleus accumbens shell may be setting the “gain” for affective or hedonic experience. Moreover, CREB overexpression in this specific tissue compartment *increases* immobility during swim stress. Changing levels of CREB in particular functional circuits may lead to vastly different phenotypes [100], and regional analyses of CREB levels are necessary to understand its function in depression. This highlights the importance of regional specificity in understanding the function of key neuroplastic modulators in depression [101].

18.2.9 Excitatory Amino Acid Receptors and Transporters in Neurons and Glia

Most neurons as well as glia have cell surface excitatory amino acid (EAA) receptors. Glutamate and other EAAs operate via four distinct classes of receptors. These include three heterogenous classes of ionotropic EAA receptors (iGluRs) [N-methyl-D-aspartate (NMDA), AMPA, and kainate receptors (KAs)] and a heterogenous class of G-protein-coupled EAA receptors (mGluRs). All of these receptors are thought to have an important role in CNS signaling in neurons and glia and as such are important therapeutic targets in neuropsychiatric diseases [102].

Cloning of the iGluRs and the mGluRs has been the basis for a dramatic expansion of research in the EAA receptor arena. At present, six NMDA receptor subunits (NR1, NR2A–2D, and NR3A) have been cloned, four AMPA receptor subunits (iGluR1–4) have been characterized, and five subunits for the KA receptors (iGluR5–7, KA1, and KA2) have been discovered [103].

The dynamic balance of glutamate is very sensitive to alterations in energy supply, and a highly efficient transport system protects neurons against the neurotoxic effects

of glutamate (reviewed in [104]). Glutamate transporters are energy dependent and essentially are the only mechanism to remove glutamate from the synapse. Thus, the brain is thermodynamically metastable due to the large amounts of toxic intracellular glutamate held in check via energy-demanding processes. Inhibition of glutamate uptake by neurons and astrocytes results in rapid buildup of glutamate, for instance, in ischemia. A *deleterious network hypothesis* has been proposed in which activation of glutamate receptors can lead to increasing levels of excitatory neurotransmitter signaling, higher energy demands, and increased production of toxic free radicals [104].

Synaptic spine dimensions are also regulated by glutamate receptor activity. The structural organization of various synapses and their relationship to astrocytes and glutamate transporters (GLT1 and GLAST) control levels of glutamate and thus activity. It has been shown in the supraoptic nucleus of the hypothalamus that there is a plastic restructuring of the neuropil leading to withdrawal of astrocytic processes at glutamatergic synapses which alters glutamate levels and glutamate release via activation of metabotropic glutamate receptors [105]. The relationship between glia and the synapse is mediated, at least for Bergmann glia that ensheath Purkinje cells, by AMPA receptor-mediated processes [106]. Crosstalk between synapses through spillover of glutamate may also have a role in understanding changes in excitability and neurotoxic effects. For instance, glial glutamate transporters in some instances may be poorly oriented in the hippocampus to deter crosstalk between nearby synapses due to limited three-dimensional astrocytic process coverage between them [107]. This could help explain particular vulnerability of the hippocampus to excitotoxicity in stress-related processes such as depression [108]. S100 β is a calcium binding protein primarily expressed by astrocytes and after secretion has a neurotrophic role.

Trophic substances released by astrocytes may be important for the maintenance of neuronal health. S100 β has been proposed as a neurotrophic factor that may protect neurons against glutamatergic toxicity [109], although high levels of glutamate can also reduce S100 β production, further reducing neuronal resilience [110], and too great an increase in S100 β can also be deleterious. S100 β is released in response to stimulation of 5-HT_{1A} receptors such that serotonergic antidepressants could have therapeutic action via this novel mechanism of increasing levels of S100 β . Recent imaging data suggest that antidepressants may protect against a reduction in hippocampal volume observed in depression. Recent advances using ¹³C nuclear magnetic resonance spectroscopy following acetate infusion indicate that glutamate/glutamine cycling between astroglia and neurons is the central pathway to replenish neuronal glutamate stores, and this imaging technique may be used in the future to study the role of glutamate signaling in depression [103].

18.2.10 Clinical Evidence for NMDA Receptors in Mood Disorders

Evidence for abnormal glutamatergic neurotransmission involvement in mood disorders can be traced back to the 1950s when the antitubercular drug D-cycloserine, a partial agonist at the NMDA glycine allosteric modulatory site, was observed to have mood-elevating effects. Moreover, amantadine is a low-affinity uncompetitive antagonist of NMDA receptors, and case reports and studies indicate that this antiviral agent has antidepressant activity in unipolar and bipolar depressed patients and in depressed subjects with Parkinson's disease [111].

The antiepileptic drug lamotrigine is widely prescribed for its antidepressant and mood-stabilizing effects in the treatment of bipolar depression, and it has been suggested that reducing pathological glutamatergic activity may be the mechanism of action [112–116]. These same agents are effective in several neurological conditions that may involve excessive activation of glutamate receptors in addition to convulsive disorders (e.g., opiate dependence, neuropathic pain, ischemia, and traumatic brain injury). Potent noncompetitive NMDA antagonists such as MK801 or ketamine may with prolonged exposure cause cortical neurotoxicity and acutely result in psychosis. A proposed mechanism is via putative NMDA receptor hypofunction that results indirectly (via GABA interneurons) in disinhibition of critical ascending glutamatergic (thalamocortical) and cholinergic (basal forebrain-cortical) pathways that may result in excessive activity at AMPA and kainate receptors [117, 118].

The channel-blocking NMDA antagonist ketamine has been observed to have rapid (within 72 h) antidepressant effects distinct from its euphorogenic effects [119], and lamotrigine has been observed to prevent the psychotic-like effects of the NMDA antagonist ketamine in normal humans [120]. Farber and colleagues have proposed that anticonvulsants may be effective by dampening voltage-gated sodium channel activity required for action potential initiation and thereby limiting activity in disinhibited ascending pathways. However, such reasoning is unlikely to fully explain the differences in psychotropic and neuroprotective effects between anticonvulsant agents [121]. For example, recent evidence suggests that the antidepressant lamotrigine selectively reduces action potential generation in dendrites without having a similar effect at the soma and that this is related to an increase in the “hyperpolarization-activated cation current (I_h), a voltage-gated current predominantly found in dendrites” [122, p. 21]. Lamotrigine may also have a portion of its therapeutic effects in epilepsy and depression via potentiation of a hyperpolarizing K⁺ current [123].

18.3 CONCLUSIONS

Despite the large number of potential novel drug targets, there is concern that there is a slowdown in the development of truly innovative drugs for many conditions. In the area of affective disorders, some of this difficulty reflects the high rate of negative and failed trials, related in part to the tremendous genetic and phenotypic heterogeneity in depression, and the lack of biological markers to guide drug development. Existing or novel endophenotypes that reduce disorders to discrete component units and ultimately fundamental units linked to pathophysiology may help move translational research forward. Clinical and genomic approaches are needed to clinically subgroup patients more precisely. Refinement of measurement tools including imaging techniques may lead to the definition of new endpoints, and biomarkers may be developed based on dissected components of current consensus syndromes that measure disease state with accuracy and objectivity. There is great promise and investment in the idea of high-throughput screening of targets determined either by evaluating disease-linked susceptibility genes and proteins (i.e., the candidate target approach) or via the open-target approach using differential display (e.g., DNA expression chips) and proteomics with postmortem samples from patients (or from animals treated with psychotropic agents). DNA databases and bioinformatics

approaches may also be used to uncover motifs or domains that have features suggestive of disease relevance [124]. Characterizing drug target effects on neurotransmitters, transcription factor levels, or brain circuitry implicated in depression can then validate novel drug targets identified. Also, if drugs with known psychotropic effects interact or regulate expression of a candidate gene or protein, this would provide further confirmation of the target's relevance.

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19

NEUROTROPHIC FACTORS IN ETIOLOGY AND TREATMENT OF MOOD DISORDERS

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19.1	Introduction	790
19.2	Neurotrophic Factors and Intracellular Signaling Cascades	791
19.2.1	Nerve Growth Factor Family	791
19.2.2	Other Classes of Neurotrophic/Growth Factors	792
19.2.2.1	Vascular Endothelial Growth Factor (VEGF)	792
19.2.2.2	Fibroblast Growth Factor (FGF)	793
19.2.2.3	Insulin and Insulin-Like Growth Factor (IGF-1)	793
19.2.2.4	Transforming Growth Factor- β (TGF- β)	793
19.3	Influence of Stress on Neurotrophic and Growth Factors	794
19.3.1	Influence of Stress on BDNF and Other Neurotrophic Factors	794
19.3.2	Stress Can Lead to Long-Lasting Neurotrophic Factor Alterations	794
19.3.3	Mechanisms Underlying Regulation of BDNF by Stress	795
19.3.3.1	Adrenal Glucocorticoids	796
19.3.3.2	5-HT Receptors	797
19.3.3.3	Cytokines: Interleukin-1 β (IL-1 β)	797
19.3.4	Influence of Stress on VEGF	797
19.4	Influence of Stress on Adult Neurogenesis and Morphology	797
19.4.1	Influence of Stress on Adult Neurogenesis	798
19.4.1.1	Neurogenesis in Adult Hippocampus	798
19.4.1.2	Stress Decreases Adult Neurogenesis	798
19.4.1.3	Mechanisms Underlying Decreased Neurogenesis	798
19.4.2	Influence of Stress on Glial Proliferation	800
19.4.3	Influence of Stress on Neuronal Morphology	800
19.5	Neurotrophic Factors in Depressed Patients	800
19.5.1	Levels of BDNF in Postmortem Tissue	800
19.5.2	Levels of BDNF in Serum	801
19.5.3	Levels of FGF in Postmortem Tissue	802
19.6	Structural and Cellular Alterations in Depressed Patients	802
19.6.1	Brain Imaging Studies of Depressed Patients	802
19.6.2	Postmortem, Morphometric Studies of Depressed Patients	802

19.7	Genetic Studies of BDNF in Mood Disorders	803
19.8	Influence of Antidepressant Treatment on Neurotrophic Factor Expression	804
19.8.1	Antidepressant Treatment Increases the Expression of BDNF	804
19.8.2	Mechanisms for Regulation of BDNF	804
19.8.3	Antidepressant Treatment Influences Other Neurotrophic Factor Systems	805
19.9	Influence of Antidepressant Treatment on Adult Neurogenesis	806
19.9.1	Antidepressant Treatment Increases Adult Neurogenesis	806
19.9.2	Mechanisms Underlying Antidepressant Regulation of Adult Neurogenesis	806
19.9.3	Influence of Antidepressant Treatment on Glial Proliferation	807
19.9.4	BDNF Increases Sprouting of 5-HT Neurons	807
19.10	Behavioral Effects of Neurotrophic Factors	808
19.10.1	Influence of BDNF in Models of Depression	808
19.10.2	Influence of Other Factors in Models of Depression	809
19.11	Summary and Conclusions	809
	Acknowledgments	809
	References	809

19.1 INTRODUCTION

The first theories of depression were based on the discovery that drugs with antidepressant efficacy could influence synaptic levels of monoamines, most notably serotonin (5-HT) and norepinephrine (NE). This included the monoamine oxidase inhibitors that block the metabolism of 5-HT and NE and the tricyclic antidepressants that block the reuptake of monoamines. These findings resulted in the monoamine hypothesis, which stated that antidepressant activity was due to an increase in levels of 5-HT and NE and that depression was due to abnormally low levels of these neurotransmitters. The 5-HT and NE systems have been and continue to be the focus of intense basic and clinical investigations into the neurobiology of depression. However, the time lag in the therapeutic response and the lack of effect of acute monoamine depletion on mood suggest that there are additional factors involved in the etiology and treatment of depression [1].

The requirement for long-term treatment to produce a therapeutic response has resulted in the widely held hypothesis that adaptations or neural plasticity play a major role in the actions of antidepressants [2–4]. This hypothesis posits that the increase in levels of 5-HT and NE leads to regulation of receptor-coupled intracellular signaling pathways and regulation of gene expression that ultimately control neuronal function that underlies behavioral alterations. There are a number of adaptations that have been identified, but one that has gained increasing interest is the regulation of neurotrophic factor expression and related signaling [1, 5]. This hypothesis is based on a combination of timely basic and clinical research studies of stress, depression, and antidepressant responses in animal models and humans over the past 10 years. This is meant to be not a complete review of the literature but rather an overview of studies relevant to a neurotrophic hypothesis of depression/antidepressant response.

19.2 NEUROTROPHIC FACTORS AND INTRACELLULAR SIGNALING CASCADES

One of the most highly studied growth factor systems in the central nervous system is the nerve growth factor (NGF) family, and the major focus of this chapter is on one of the members of this family, brain-derived neurotrophic factor (BDNF). However, there are many other important neurotrophic/growth factor systems that are now being examined in the context of complex normal and abnormal neuronal and behavior functions (Table 19.1). A brief review of the major neurotrophic/growth factor systems is provided in this section, particularly where there is information that relates these factors to stress, depression, and antidepressant responses (see also Chapter 8 in Volume III of this handbook). One of the key features that link these different growth factor systems is the activation of intracellular tyrosine kinase signaling pathways (Table 19.1). This could account in part for the overlap and function of these diverse growth factor systems in the actions of stress and antidepressant treatment.

19.2.1 Nerve Growth Factor Family

This neurotrophic factor family is comprised of NGF, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4/5) (Table 19.1). These factors were originally identified for their role in the development of the central nervous system, including neurogenesis, differentiation, and survival. However, members of the neurotrophic factor family are also expressed in the adult brain, the expression of certain factors (e.g., BDNF) is dependent on neuronal activity, and these factors can modulate the survival and function of adult neurons [6]. The receptors, referred to as Trk (derived from troponin/receptor kinases), have an extracellular binding domain and an intracellular tyrosine kinase domain. There are three different receptors, TrkA, TrkB, and TrkC, that bind NGF, BDNF, and NT-3 and NT4/5, respectively, although NT-3 can also bind TrkB.

Binding of a neurotrophic factor ligand to a Trk receptor leads to activation and autophosphorylation of the intracellular tyrosine kinase domain, resulting in association with proteins containing a Src homology (SH) domain. This can lead

TABLE 19.1 Neurotrophic and Growth Factor Families, Receptors, and Kinases

Family	Members	Receptor/Kinase
Nerve growth factor (NGF)	NGF, BDNF, NT-3, NT-4/5	TrkA-C, tyrosine kinases
Vascular endothelial growth factor (VEGF)	VEGF-A,B,C,D,E, PlGF, PDGF-A,B	R1 (Flt-1), R2 (Flk-1), R3 (Flt-4), tyrosine kinases
Fibroblast growth factor (FGF)	FGF2, many others (FGF1–23)	FGFR1–4, tyrosine kinases
Insulin-like growth factor (IGF-1)	Insulin, IGF-1, IGF-2	IGF-1R, tyrosine kinase
Transforming growth factor- β (TGF- β)	TGF- β 1–5, activins/inhibins	T- β RI, RII, Ser/Thr kinases, activinRI, RII, Ser/Thr kinases

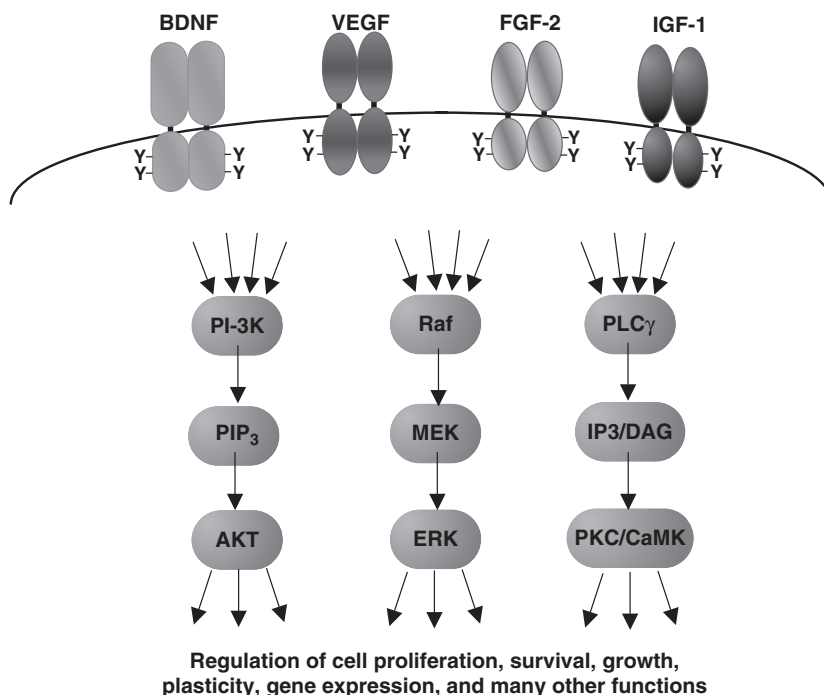


Figure 19.1 BDNF, VEGF, FGF-2, and IGF-1 can activate the same signal transduction cascades. These factors bind to receptors that have an extracellular binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Binding of each factor to its receptor results in autophosphorylation of tyrosine residues (Y). This in turn can lead to activation of one or more signaling cascades as indicated by multiple arrows, including the PI-3K, ERK, and PLC- γ pathways. (See color insert.)

to activation of different signal transduction systems depending on the cell type (Fig. 19.1). These include the Shc/Ras extracellular regulated kinase (ERK) pathway [also referred to as the microtubule-associated protein kinase (MAPK) cascade], phospholipase C- γ (PLC- γ) and protein kinase C (PKC), phosphatidylinositol-3'-OH kinase (PI-3 K), and protein kinase B (AKT).

19.2.2 Other Classes of Neurotrophic/Growth Factors

19.2.2.1 Vascular Endothelial Growth Factor (VEGF). VEGF exerts powerful effects on vascular permeability but also induces angiogenesis and endothelial cell proliferation [7]. VEGF is a heparin binding glycoprotein that is secreted as a 45-kDa homodimer. There are five different members of the VEGF family, VEGFA–E (Table 19.1). Related growth factors include placental growth factor (PlGF) and platelet-derived growth factors (PDGF) A and B. These ligands bind to one of several different receptors, VEGFR1 (also known as Flt-1), VEGFR2 (also known as Flk-1 or KDR), and VEGFR3 (also known as Flt-4). The neuropilin receptors 1 and 2 are also related to the VEGF receptors. The VEGF receptors have an intracellular tyrosine kinase domain and multiple immunoglobulin G (IgG)-like extracellular

domains. The VEGF receptors couple to multiple intracellular cascades via interactions with many of the same signaling proteins activated by the neurotrophin-Trk receptors, including Shc/Ras-ERK, PLC-g-PKC, and PI-3K-AKT (Fig. 19.1).

19.2.2.2 Fibroblast Growth Factor (FGF). The FGF family consists of 23 different members, 10 of which are expressed in the brain [8] (Table 19.1). These factors play a role in neuronal development, including neurogenesis, differentiation, axonal branching, and neuronal survival. In addition, the FGF family members are involved in the adult neuronal function, most prominently neuronal repair, as well as in learning and memory. There are four FGF receptors, R1–4, which have three extracellular Ig-like domains and an intracellular tyrosine kinase domain. FGF binding leads to activation and autophosphorylation of the receptor. This leads to interactions with SH2 domains of signaling cascades and activation of intracellular signaling proteins, including src and PLC- γ -PKC as well as Shc/Ras-ERK and Crk (Fig. 19.1).

19.2.2.3 Insulin and Insulin-Like Growth Factor (IGF-1). This family of growth factors is comprised of insulin, IGF-1, and IGF-2 (Table 19.1). These factors are important mediators of growth as well as metabolism in both the peripheral and central nervous systems [9]. IGF-1 is synthesized in peripheral tissues, primarily the liver, and is also found and synthesized in most parts of the central nervous system [10]. Insulin and IGF-1 are potent neurotrophic factors for neurons and glia during development but also regulate synaptic plasticity, neuronal survival, and protection in the adult brain. IGF-1 also regulates oligodendrocyte survival as well as myelination, and insulin modulates food intake, glucose homeostasis, and growth. The function of IGF-1 is also controlled by levels of IGF-1 binding proteins that are expressed in the brain and peripheral tissues. The receptors for insulin and IGF-1 have an intracellular tyrosine kinase domain, and binding leads to activation and autophosphorylation of the receptor. This in turn results in association with insulin receptor substrate (IRS-1) and activation of the PI-3K-Akt pathway or Shc/Ras-ERK and PLC- γ -PKC (Fig. 19.1).

19.2.2.4 Transforming Growth Factor- β (TGF- β). TGF- β and related family members are multifunctional growth factors that play a significant role in development as well as neuroprotection and repair in the adult brain [11, 12]. There are several subfamilies, including the TGF- β (1–5), activins/inhibins, bone morphogenic proteins (BMP1–13), growth differentiation factors, (GDF1–10), and glial-derived neurotrophic factor (GDNF, artemin, neurturin, and persephin) (Table 19.1). The general mechanism for activation of intracellular signaling is the association of the receptors with another protein that contains an intracellular kinase domain. In most cases this is a serine/threonine kinase. Activation and phosphorylation of the receptor-associated proteins leads to phosphorylation of Smad2 and Smad3, which together with Smad4 regulate gene transcription [13]. The GDNF family is more distantly related and the associated receptor, ret, is a tyrosine kinase associated protein that can lead to regulation of a similar set of intracellular cascades as discussed for other tyrosine kinases.

Recent studies demonstrate that antidepressant treatment increases the expression of activin β A and/or increases Smad2 phosphorylation [14]. Moreover, infusions of activin A into the hippocampus produce an antidepressant response in the behavioral models

of depression. These findings provide support for a role of TGF- β /activin in antidepressant responses and further studies of this interesting family of growth factors.

19.3 INFLUENCE OF STRESS ON NEUROTROPHIC AND GROWTH FACTORS

One of the most challenging issues in efforts to study the neurobiology of complex psychiatric illnesses is to develop good animal models. This is particularly true for mood disorders, where it is extremely difficult, if not impossible, to assess the emotional state of an animal. Although by no means perfect, studies of stress, which is known to precipitate or exacerbate depression and other disorders [15, 16], have been very useful and productive (see reviews in [16a–d]). Studies demonstrating that stress decreases neurotrophic factor expression in limbic brain regions associated with depression have played a key role in the formation of a neurotrophic hypothesis of depression. The relevance of these findings is highlighted by studies in depressed patients, opposing actions of antidepressant treatment, and behavioral studies of these factors (see Table 19.2 for a summary).

19.3.1 Influence of Stress on BDNF and Other Neurotrophic Factors

The first studies of stress were focused on the neurotrophic factor family, including NGF, BDNF, and NT-3. This was based largely on earlier studies demonstrating that the expression of these factors was very dynamic and regulated by a variety of stimuli. The studies of stress have focused on the hippocampus, a limbic brain region that has been implicated in mood disorders and that expresses high levels of receptors for adrenal glucocorticoid receptors. Smith and colleagues [23] were the first to report that exposure to stress causes a rapid and robust downregulation of BDNF in the hippocampus. Exposure to immobilization stress for only 1 to 2 h decreases the expression of BDNF in the dentate gyrus granule cell layer and the CA3 pyramidal cell layer, two subfields of the hippocampus that are influenced at the structural level by stress (see below). A similar effect of immobilization stress is observed in female rodents [17]. In addition to immobilization stress, BDNF expression is also decreased by chronic unpredictable stress [18], swim stress [19], footshock [20], and early maternal deprivation [21]. These studies provide evidence that stress could alter neuronal survival and function via regulation of neurotrophic factor expression.

Stress is also reported to influence the expression of other members of the neurotrophic factor family. Long-term immobilization stress (8 h) decreases the expression of NGF and NT-3 in the hippocampus [22]. This study also found decreased levels of the neurotrophic factor receptors TrkA, TrkB, and TrkC. In contrast, Smith and colleagues [23] did not observe a decrease in levels of NT-3 or the neurotrophic factor receptors. In fact, they reported that levels of NT-3 are increased in the hippocampus by repeated exposure to stress. Another study also reports that stress increases levels of NT-3 in locus ceruleus noradrenergic cell bodies [24].

19.3.2 Stress Can Lead to Long-Lasting Neurotrophic Factor Alterations

In addition to acute effects, exposure to stress can produce long-lasting changes in the expression of BDNF. This is demonstrated most effectively by studies at early

TABLE 19.2 Regulation of BDNF by Stress, Depression, and Antidepressant Treatment

Treatment	Effect
Rodent studies, hippocampus	
Stress	
Immobilization	Decrease
Footshock	Decrease
Unpredictable	Decrease
Social isolation	Decrease
Maternal deprivation	Decrease
Corticosterone ^a	Decrease
Antidepressant	
ECS	Increase
MAOI	Increase
SSRI	Increase
NESRI	Increase
TMS	Increase
Exercise ^b	Increase
Human studies	
Depression, postmortem hippocampus	
Depressed	Decrease
Depressed + antidepressant	Increase
Depression, blood	
Depressed	Decrease
Depressed + antidepressant	Increase

Abbreviations: ECS, electroconvulsive seizures; MAOI, monoamine oxidase inhibitor; SSRI, 5-HT selective reuptake inhibitor; NESRI, norepinephrine selective reuptake inhibitor; TMS, transcranial magnetic stimulation.

^aCorticosterone treatment which simulates stress levels of this adrenal glucocorticoid also decreases BDNF.

^bExercise is also reported to increase levels of BDNF and produces an antidepressant effect in models of depression.

postnatal time points. Animals exposed to early maternal deprivation (24 h on postnatal day 9) exhibit deficits in the expression of BDNF as adults [21]. Another postnatal maternal deprivation paradigm (3 h from days 2 to 14) is reported to decrease BDNF in the prefrontal cortex [19]. Early postnatal care (the amount of licking and grooming of pups) also regulates the expression of BDNF in the adult [25]. Exposure to a tone previously paired with a footshock decreases the expression of BDNF in the hippocampus [20]. These studies demonstrate how stress and prior experience can lead to long-term alterations in the expression of BDNF that could influence neuronal function and have consequences throughout the life of an animal (see below).

19.3.3 Mechanisms Underlying Regulation of BDNF by Stress

Stress can influence a number of endocrine, neurotransmitter, and cytokine systems that could contribute to the regulation of BDNF in the brain. This section provides a brief overview of the literature addressing this issue (see Fig. 19.2 for a summary).

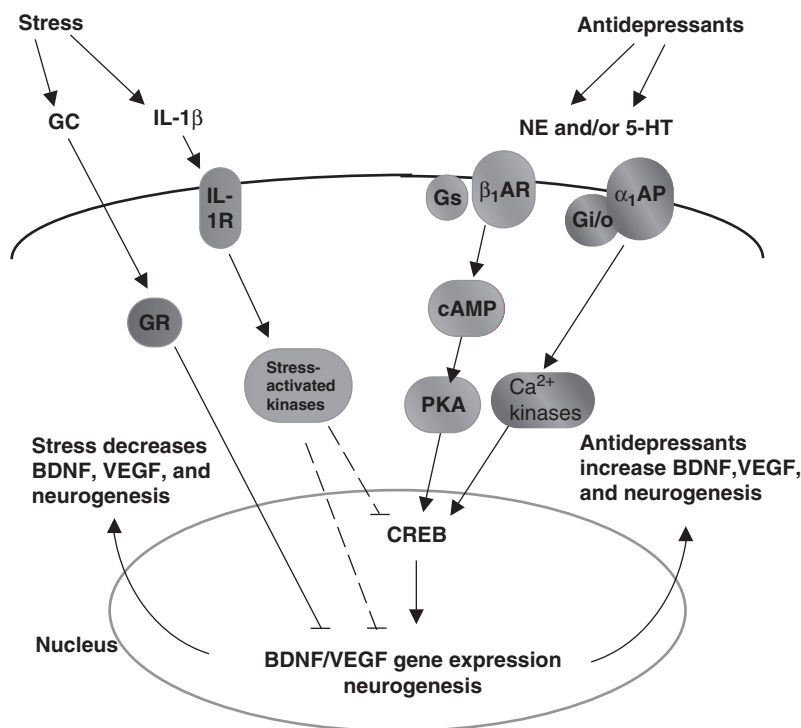


Figure 19.2 Mechanisms underlying regulation of BDNF and VEGF by stress and antidepressant treatment. The downregulation of BDNF and VEGF by stress occurs via elevated levels of glucocorticoids (GCs) and activation of the GC receptor (GR), which can directly regulate the expression of BDNF. Stress also increases levels of interleukin-1 β (IL-1 β), which can decrease BDNF, although the intracellular pathways responsible for this effect have not been determined (indicated by dashed lines). The role of GC and IL-1 β in the downregulation of VEGF has not been determined. In contrast, antidepressant treatment upregulates the cyclic adenosine monophosphate (cAMP) cascade, including cAMP-dependent protein kinase (PKA) and cAMP response element binding protein (CREB), which increases BDNF and VEGF gene transcription. CREB can also be phosphorylated by Ca²⁺-dependent kinases, such as Ca²⁺/calmodulin-dependent protein kinase. These kinases can be activated by NE and 5-HT receptors, including the β -adrenergic 1 (β_1 AR) and α_1 -adrenergic receptors and their respective G-protein subunits, the stimulatory (G_s) and inhibitory ($G_{i/o}$) G proteins. (See color insert.)

19.3.3.1 Adrenal Glucocorticoids. The hallmark endocrine response to stress is activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in elevated levels of adrenal glucocorticoids. The receptors for glucocorticoids (GRs) as well as mineralocorticoids (MRs) are expressed in high levels in the hippocampus, and because of the lower affinity of GR only stress-induced levels of glucocorticoids activate the GRs. A role for glucocorticoids in the actions of stress is supported by studies demonstrating that administration of corticosterone, the rodent equivalent of human cortisol, decreases the expression of BDNF [23, 26, 27]. In contrast, removal of adrenal glands, where corticosterone is synthesized, leads to up-regulation of BDNF [26, 28]. However, the role of glucocorticoids in the action of stress appears to be more complicated. In adrenalectomized animals receiving a low dose of

corticosterone replacement exposure to stress still decreases BDNF expression [23], indicating that other factors are required for the effects of stress.

19.3.3.2 5-HT Receptors. Reports that acute stress increases serotonin neurotransmission have led to studies of the role of 5-HT receptors in the regulation of neurotrophic factors. Administration of a 5-HT₂, but not 5-HT₁, receptor agonist results in a rapid and robust downregulation of BDNF expression in the hippocampus of male [29] or female rats [17], similar to the effects of stress. The actions of a nonselective 5-HT₂ receptor agonist are blocked by a selective 5-HT_{2A}, but not by a 5-HT_{2C} antagonist [29]. In addition, 5-HT_{2A} receptor blockade reverses the downregulation of BDNF resulting from exposure to immobilization stress [29]. The cellular mechanisms underlying the regulation of BDNF by 5-HT_{2A} receptors can be explained by the presence of these receptors on GABAergic interneurons in the granule cell layer. Activation of these receptors increases GABA-mediated inhibitory postsynaptic potentials (IPSPs) in granule cells that could underlie a decrease in activity-dependent regulation of BDNF.

19.3.3.3 Cytokines: Interleukin-1 β (IL-1 β). Stress can also lead to alterations in levels of cytokines, most notably increased levels of IL-1 β , raising the possibility that the downregulation of BDNF is mediated by this cytokine (Fig. 19.1). This hypothesis is supported by a study demonstrating that administration of an antagonist of IL-1 β blocks the downregulation of BDNF in response to stress [30]. The actions of IL-1 β may also be explained by an effect on cell activity. IL-1 β decreases the release of glutamate as well as Ca²⁺ influx [31], which could lead to a reduction in the activity-dependent expression of BDNF in the hippocampus.

19.3.4 Influence of Stress on VEGF

A recent study has demonstrated that stress can also influence the expression of VEGF. VEGF is known primarily for regulation of endothelial cell proliferation and angiogenesis, but it also has neurotrophic and neuroprotective effects. In addition, VEGF increases adult neurogenesis in the hippocampus and has been implicated in the vascular niche hypothesis of adult neurogenesis [32]. For these reasons VEGF has been studied in models of stress as well as antidepressant treatment (see below). Exposure to chronic unpredictable stress (a combination of immobilization, swim stress, crowding or isolation, and vibration) decreases the expression of VEGF protein in the granule cell layer and hilar region of the hippocampus [33]. The expression of the VEGF receptor type 2 is also decreased in the granule cell layer and hilus in this chronic stress paradigm. The influence of stress on VEGF may be due to adrenal glucocorticoids, which downregulate the expression of VEGF as well as the type 2 receptor in certain tissues [34, 35]. Further studies will be required to determine if glucocorticoids underlie the stress-induced VEGF decrease in the hippocampus.

19.4 INFLUENCE OF STRESS ON ADULT NEUROGENESIS AND MORPHOLOGY

Altered neurotrophic factor expression as well as alterations of neuroendocrine and neurotransmitter systems could result in functional effects at the cellular level in

response to acute and chronic stress. In this section, the alterations in the proliferation of cells in the adult brain as well as morphological changes in dendrite formation are discussed, and the potential roles of neurotrophic factors in these responses to stress are examined. A more complete review of neurogenesis is provided in Chapter 20 of this volume as well as Chapter 7 in Volume III of this handbook.

19.4.1 Influence of Stress on Adult Neurogenesis

19.4.1.1 Neurogenesis in Adult Hippocampus. Although neurogenesis largely takes place during the developmental stage, there are two regions in the brain, the hippocampus and the subventricular zone, where new neuronal birth continues to take place into adulthood and even old age [36, 37]. In the hippocampus the neural progenitor cells are located in the subgranular zone between the granule cell layer and the hilus. These cells divide and give rise to cells that differentiate and mature into neurons that migrate into the granule cell layer of the hippocampus (Fig. 19.3). These newborn granule cells take on the appearance and function of mature granule cells over the course of four to eight weeks, sending axons to the CA3 pyramidal cell layer and dendrites to the molecular layer and exhibiting electrophysiological characteristics of mature granule cells [37]. Adult neurogenesis has been observed in a variety of animals, including humans, well into old age.

19.4.1.2 Stress Decreases Adult Neurogenesis. One of the most striking characteristics of adult neurogenesis in the hippocampus is the dynamic nature of this process. The rate of proliferation and/or the survival of newborn cells can be regulated in either a positive or negative manner by a variety of stimuli. For example, adult neurogenesis is increased by enriched environment, exercise, hippocampal-dependent learning, and antidepressant treatment (see below) [38, 39]. In contrast, treatment with drugs of abuse or exposure to stress decreases adult neurogenesis (see Fig. 19.3). The ability of adult neurogenesis to be up- or downregulated by positive and negative stimuli suggests that it is a form of neural plasticity that contributes to the mechanisms underlying long-term changes in the brain.

The downregulation of adult neurogenesis has been observed with several different types of stress [5, 16a], including intruder stress [40], predator stress [41], maternal separation stress [42], chronic mild stress [43], and footshock [44, 45]. This effect is observed in many different species, including mice, rats, tree shrews, marmosets, and rhesus monkeys, indicating that it is a widespread response to stress [5]. In the learned helplessness model of depression there is a long-lasting downregulation of adult neurogenesis after exposure to inescapable but not escapable stress that correlates with the behavioral despair in this model [44]. Moreover, antidepressant treatment reverses the neurogenesis deficit as well as the behavioral despair [44]. However, another study has reported that decreased neurogenesis does not correlate with behavior in the learned helplessness model [45]. The correlation between neurogenesis and behavioral responses is further discussed in Section 19.8.

19.4.1.3 Mechanisms Underlying Decreased Neurogenesis. The stress responsive HPA axis has also been implicated in the regulation of adult neurogenesis. Adrenal glucocorticoid administration decreases adult neurogenesis in the hippocampus [46] and adrenalectomy blocks the downregulation of neurogenesis in response to stress

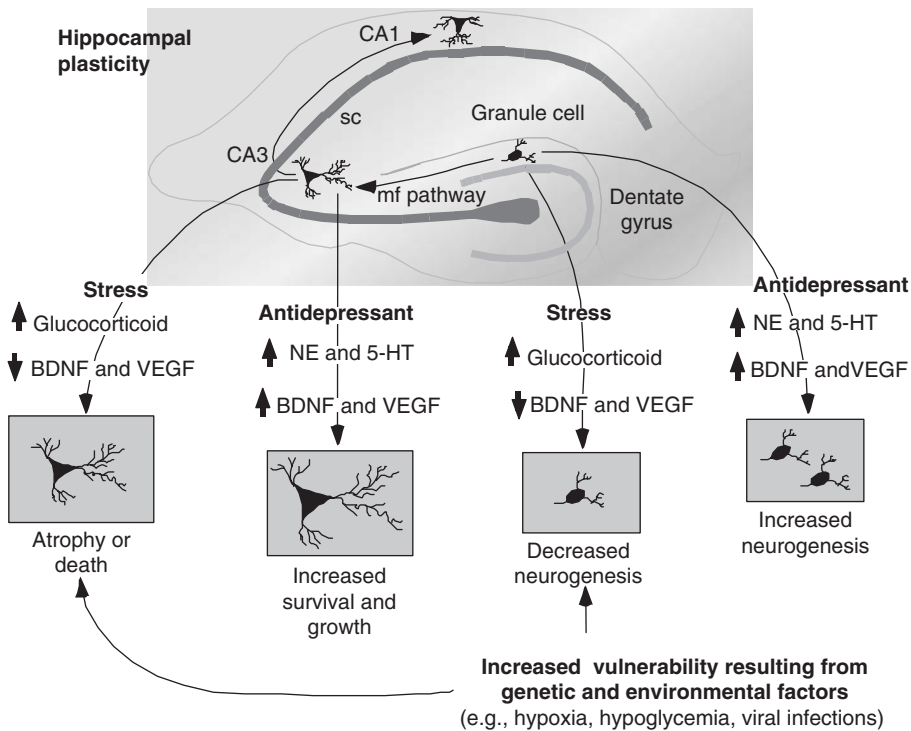


Figure 19.3 Influence of stress and antidepressant treatment on hippocampal plasticity: Regulation of adult neurogenesis and neuronal remodeling/atrophy. Exposure to stress can lead to at least two distinct cellular events in the hippocampus: a decrease in the number and length of apical dendrites of CA3 pyramidal cells and a decrease in the proliferation of new neurons in the granule cell layer. In contrast, antidepressant treatment can block or reverse the effects of stress and increase neurogenesis in the granule cell layer of adult hippocampus. The effects of stress are mediated by elevated levels of adrenal glucocorticoids and activation of *N*-methyl-D-aspartate (NMDA) receptors (not shown). Downregulation of BDNF and VEGF may also contribute to the deleterious effects of stress. The actions of antidepressant treatment are thought to occur via increased levels of NE and 5-HT, which in turn lead to increased levels of BDNF and VEGF. (See color insert.)

[5, 16a, 41]. There are several neurotransmitters or factors that could be acting downstream of glucocorticoids. Activation of *N*-methyl-D-aspartate (NMDA) excitatory amino acid receptors decreases and NMDA receptor antagonists increase adult neurogenesis [47, 48].

As discussed earlier, both BDNF and VEGF are decreased by stress and adrenal glucocorticoids and could contribute to the downregulation of neurogenesis that occurs after exposure to stress (Fig. 19.3). Infusions of BDNF into the lateral ventricles increase neurogenesis in a number of brain structures, but there is no increase in the hippocampus [49, 50]. This could be due to insufficient diffusion of BDNF to the hippocampus. Viral expression or infusions of recombinant VEGF increase adult neurogenesis in the hippocampus [51, 52]. Additional studies will be needed to determine if BDNF and/or VEGF can rescue the downregulation of

neurogenesis that occurs in response to stress. The role of these factors in the actions of antidepressant treatment is also discussed below.

19.4.2 Influence of Stress on Glial Proliferation

In addition to regulation of neurogenesis in adult hippocampus, stress also influences the proliferation of glia. This may be relevant to the neurobiology of depression because of the reports of decreased glia in the prefrontal cortex and other limbic structures of depressed patients (see below). Exposure to repeated unpredictable mild stress (14 days) but not acute stress decreases the number of newborn cells in the prefrontal cortex of adult rats (G. Valentine, M. Banasr, and R. S. Duman, Yale University, November 2006, unpublished data). Newborn cells in the prefrontal cortex express markers of oligodendrocytes and endothelial cells as well as a population of unidentified cells. Under the conditions of these studies there were no newborn cells in the cerebral cortex expressing neuronal markers. A previous study has demonstrated that chronic administration of corticosterone decreases glial proliferation in brain [53], suggesting that increased glucocorticoid levels underlie the decrease in cell proliferation observed in the prefrontal cortex.

19.4.3 Influence of Stress on Neuronal Morphology

The regulation of neuronal morphology, including alterations in the number and length of neuronal processes, represents another form of structural plasticity in the nervous system, and stress is reported to alter the length of neuronal dendrites. Chronic restraint stress (18 days) decreases the number and length of the apical dendrites of the CA3 pyramidal neurons in the hippocampus ([54]; also see [16a]) (Fig. 19.3). This atrophy is reversible over the course of several weeks when the animals are no longer exposed to stress. Chronic administration of corticosterone produces a similar effect, indicating that the effects of stress occur via increased levels of adrenal glucocorticoids [16a]. In addition, blockade of NMDA receptors blocks the atrophy of CA3 pyramidal cells, suggesting a role for glutamate and this receptor subtype. The atrophy of hippocampal neurons in combination with decreased neurogenesis could contribute to the reduction in hippocampal volume observed in depressed patients.

19.5 NEUROTROPHIC FACTORS IN DEPRESSED PATIENTS

The results of rodent studies demonstrate that the expression of BDNF is decreased by stress and increased by antidepressant treatment, contributing to a neurotrophic hypothesis of depression. This section reviews the clinical studies in humans, in both postmortem brain tissue and blood, designed to directly test this hypothesis (see Table 19.2 for a summary). BDNF has been the focus of the majority of these studies, but it will be interesting to examine other neurotrophic and growth factors in future studies.

19.5.1 Levels of BDNF in Postmortem Tissue

The most direct approach to test the neurotrophic hypothesis is to analyze levels of BDNF in the brains of depressed patients. Unfortunately this can only be done in

postmortem tissue, which presents many limitations, including the number of patients that can be analyzed, the appropriate controls, and the interpretation of results with regard to the state or trait effects in each subject, to mention a few. However, there are now a number of postmortem studies demonstrating that levels of BDNF in the hippocampus are decreased in depressed suicide subjects relative to matched controls, or an increase in patients receiving antidepressant medication at the time of death [55–57]. One of these studies reports a similar effect in the prefrontal cortex [57]. There is also a report that one of the key BDNF-TrkB signaling kinases, ERK, is decreased in depressed patients [58].

Decreased expression of BDNF in the hippocampus and/or prefrontal cortex of depressed subjects is consistent with a neurotrophic hypothesis of depression. This decrease could be due to the stress associated with depression and sustained activation of the HPA axis. However, further studies will be needed to confirm this decrease in additional postmortem subjects and to examine the specificity of this effect to depression. There is also a report of decreased BDNF levels in prefrontal cortex of schizophrenic patients [59], suggesting that the downregulation of BDNF is not specific to depression. However, this study did not report on levels of BDNF in the hippocampus and it is possible that there are region-specific effects in different psychiatric illnesses.

19.5.2 Levels of BDNF in Serum

BDNF levels in serum are much easier to assess and can be conducted in living subjects. However, a major limitation of this approach is the relevance of BDNF in blood to levels in brain. Nevertheless, due to the compelling preclinical studies as well as brain imaging studies demonstrating reduced brain volume in depressed patients, there has been an interest in examining serum levels of BDNF. Serum BDNF in depressed patients is significantly reduced relative to matched controls, and there is a significant negative correlation with the severity of depression [60, 61]. Moreover, more recent studies have demonstrated that antidepressant treatment can either partially [62] or completely [63, 64] reverse the deficit in serum levels of BDNF in depressed patients. In a study of healthy volunteers serum BDNF is associated with depression-related personality traits [65]. Further studies will be required to determine if decreased BDNF in blood is a phenotypic marker of depression and to determine the functional relevance of altered blood levels of BDNF.

To examine the mechanisms underlying decreased serum BDNF levels in plasma, serum and blood have been determined. The major source of BDNF is platelets, although it is also stored in endothelial cells and lymphocytes. Levels of BDNF are decreased in serum and plasma but not in blood, and there is no increase in a marker of platelet activation [57]. The interpretation of these findings is that the decreased serum BDNF is related to altered mechanisms of BDNF release from platelets and not BDNF synthesis. Although speculative, it is possible that there are similar problems with the release of BDNF from neurons in the brain. However, this study does not rule out the possibility that elevated adrenal glucocorticoids or second-messenger signaling also plays a role in decreased BDNF levels in the blood.

19.5.3 Levels of FGF in Postmortem Tissue

Microarray analysis has also demonstrated alterations of the FGF system in postmortem tissue from depressed patients [66]. This study reports that the expression of several members of the FGF family, including FGF1, FGF2, and the receptors FGFR2 and FGFR3, are decreased in the dorsal lateral prefrontal cortex (DLPFC) and/or the anterior cingulate cortex of depressed patients. The effects appear to be most significant in the DLPFC where the downregulation of FGF1, FGFR2, and FGFR3 is observed in two small but separate cohorts and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). These findings are interesting in light of preclinical studies demonstrating that antidepressant treatment upregulates FGF2 in cerebral cortex and hippocampus (see below). Additional studies will be necessary to determine if similar alterations are observed in other tissue banks of depressed subjects.

19.6 STRUCTURAL AND CELLULAR ALTERATIONS IN DEPRESSED PATIENTS

A decrease in levels of neurotrophic factors could result in a reduction in the volume of certain brain structures as well as a decrease in cell number and altered morphology. Brain imaging and postmortem studies have addressed this question and provide additional evidence that is consistent with a neurotrophic hypothesis of depression. However, a direct causative relationship between neurotrophic factors and brain volume and cell morphology has not been established, and additional studies are needed to confirm these findings in depressed patients.

19.6.1 Brain Imaging Studies of Depressed Patients

Imaging studies of the hippocampus report a reduction in volume in patients with depression [67–76]. There are also negative reports, although specific measurements of hippocampus were not conducted, included the amygdala, or found altered hippocampal shape [77–79]. The magnitude of the reduction has been directly related to the length of illness [73], and antidepressant treatment reduces or even reverses the atrophy of hippocampus [72, 76]. The volume of hippocampus is also decreased in patients with posttraumatic stress disorder (PTSD) [80–84], and further studies are needed to determine if there are factors that distinguish the hippocampal changes in these two disorders.

Volumetric alterations are not limited to the hippocampus, as there are also reports of decreased volumes of the prefrontal cortex [85] and the amygdala [86], brain regions linked to altered mood, anxiety, and cognition in depressed patients. Although there is less known about the mechanisms underlying the altered volume of prefrontal cortex and amygdala, there are a number of postmortem studies examining these regions (see below).

19.6.2 Postmortem, Morphometric Studies of Depressed Patients

Although there has been extensive examination of hippocampus by brain imaging, there are relatively few studies at the cellular level. There are reports of no gross

change in cellular morphology in the hippocampus, although semiquantitative methods were used [87, 88]. Another recent study has found an increase in the density of neurons and glia in the major subfields of hippocampus, including the CA1 and CA3 pyramidal cell layers and the dentate gyrus granule cell layer, but a decrease in the size of neuronal cell bodies [89]. One possible interpretation of these findings is that the increase in cell density results from a decrease in neuropil and that this leads to a decrease in hippocampal volume. This possibility is supported by reports of decreased neuronal spine density and reduced arborization of apical dendrites in the subiculum of patients with bipolar disorder or depression [90]. Another study has reported decreased levels of synaptic proteins in the CA4 pyramidal cell layer of patients with bipolar depression [91]. Additional studies of the hippocampus and other brain structures will be needed to further define the cellular abnormalities underlying decreased hippocampal volume in depressed patients.

Studies of the prefrontal and other cortical structures have been more consistent and demonstrate a reduction in the density of glia and size of neurons. Postmortem studies from multiple laboratories have demonstrated a decrease in glial density in the dorsal lateral prefrontal cortex [92, 93], cingulate cortex [94], subgenual prefrontal cortex [95], and amygdala [96, 97]. In addition, these studies have provided evidence of a reduction in the size of neuronal cell bodies, which could result from loss of metabolic and/or neurotrophic support that is provided by glia. These effects could contribute to the reduction in the volume of the cortical regions observed in depressed patients.

19.7 GENETIC STUDIES OF BDNF IN MOOD DISORDERS

The basic research and clinical studies of BDNF as well as imaging and cell morphology studies have stimulated interest in the analysis of the BDNF gene and psychiatric illnesses. A functional polymorphism has been identified that encodes a variant of BDNF at codon 66 (*val66met*) [98]. The *met* allele results in abnormal intracellular packaging and secretion of BDNF and carriers of this allele have poorer episodic memory and lower hippocampal *N*-acetyl aspartate [98]. However, the association of the *val66met* alleles with psychiatric illness has been inconsistent. The BDNF *val* allele has been identified as a potential risk locus for bipolar disorder [99–101]. In addition, decreased hippocampal volume has been observed in bipolar patients that carry the substitution (H. Blumberg, Yale University, November 2006, personal communication). However, this association has not been observed in studies conducted in Asian populations [102, 103].

Another study of childhood-onset mood disorder did not find an association with the BDNF *val66met* alleles but did find an association with a dinucleotide repeat [104]. Another single-nucleotide polymorphism has been identified in the BDNF promoter I that results in decreased DNA binding and reduced basal promoter activity [105]. This low-activity allele was associated with reduced anxious temperament and therefore may be protective against anxiety and related illnesses. This study also reports an association of the *met66* allele with increased risk for anxiety [105]. Together these results demonstrate how the presence of different alleles can complicate the genetic analysis of BDNF polymorphisms. Additional studies will be required to further investigate the role of these as well as other novel BDNF polymorphisms in depression and other mood disorders.

19.8 INFLUENCE OF ANTIDEPRESSANT TREATMENT ON NEUROTROPHIC FACTOR EXPRESSION

In addition to studies of stress and depression, reports that antidepressant treatments upregulate levels of BDNF and other neurotrophic/growth factors have contributed to a neurotrophic hypothesis (see Table 19.2). The influence of antidepressant treatment on expression of these factors is reviewed in this section.

19.8.1 Antidepressant Treatment Increases the Expression of BDNF

Chronic administration of different classes of antidepressants is reported to increase levels of BDNF expression in the hippocampus and in some cases the cerebral cortex [106, 107]. In general the upregulation of BDNF is dependent on chronic treatment, consistent with the time course for the therapeutic response to antidepressants, and is not observed after single acute drug administration. Different classes of antidepressants, including ECS, 5-HT and NE selective reuptake inhibitors, MAOIs, and atypical antidepressants, increase BDNF expression. It is also notable that other types of treatments that have antidepressant effects, including transcranial magnetic stimulation [107a], AMPAkinetics [107b], NMDA antagonists [107c] and exercise [107d–107g], also increase the expression of BDNF. These findings are consistent with postmortem and blood studies demonstrating an increase in levels of BDNF in depressed subjects that are being treated with an antidepressant [107h–107j].

In contrast, repeated administration of non-antidepressant drugs, including cocaine or morphine, does not increase BDNF expression in the hippocampus, demonstrating the pharmacological specificity of antidepressant regulation of BDNF [107]. The typical antipsychotic haloperidol decreases BDNF, while the atypical antipsychotics clozapine and risperidone, which are known to enhance the clinical efficacy of SSRI antidepressants, increase BDNF in the hippocampus [107–109].

Although antidepressant induction of BDNF has been confirmed in a number of studies, there are reports that are inconsistent depending on the type of treatment tested. ECS and MAOI antidepressants have been found to consistently upregulate BDNF expression in the hippocampus [107e, 109a–109h]. Most of these studies have also found that 5-HT or NE selective reuptake inhibitors increase BDNF expression, but there are also a few studies that have not observed an increase with selective reuptake inhibitors [110, 111]. Similarly, mixed results have been observed with atypical antidepressants such as mianserin and tianeptine [109a, 109c, 111a].

There are a number of potential explanations for the variations that have been observed. The most likely possibilities are the length of drug treatment [110, 112, 113] and/or the dose of antidepressant used (see [113a]). The latter study found that a moderate dose of antidepressant (5 mg/kg, venlafaxine or amitriptyline) increased BDNF expression, but a higher dose (10 mg/kg) of these drugs did not [114]. The expression of BDNF is activity dependent and dynamically regulated by a variety of stimuli, and interactions of antidepressants with other environmental factors could also contribute to the outcome of drug studies. Therefore, care must be taken when designing and conducting drug studies of BDNF expression in animals.

19.8.2 Mechanisms for Regulation of BDNF

The regulation of BDNF by ECS and different classes of antidepressants indicates that there could be multiple receptor signaling pathways that regulate BDNF gene

expression. The induction by ECS can be explained by activity-dependent regulation of BDNF following neuronal depolarization and Ca^{2+} -activated signal transduction [115]. Ca^{2+} signaling leads to activation of calcium/calmodulin-stimulated protein kinase and activation of CREB. CREB is a transcription factor that can bind to cAMP response elements located on BDNF exon-specific promoters (I and III), and phosphorylation of CREB stimulates gene transcription. The BDNF gene is comprised of a total of four exon-specific promoters (I–IV) as well as one coding BDNF (exon V), and the regulatory elements of the other three promoters have not been characterized.

The influence of antidepressants that regulate synaptic levels of 5-HT and NE can also be explained by the regulation of CREB (Fig. 19.2). Chronic administration of other classes of antidepressants, including 5-HT and NE selective reuptake inhibitors and MAOIs, increase the phosphorylation and function of CREB as well as elements of the cAMP signal transduction cascade (i.e., cAMP-dependent protein kinase) that can stimulate CREB [106, 116, 117]. Additional evidence for a role of the CREB cascade is provided by studies demonstrating that rolipram, a putative antidepressant that inhibits cAMP metabolism, upregulates CREB and BDNF expression [106]. In addition, antidepressant induction of BDNF is blocked in CREB $\alpha\Delta$ mutant mice [118].

19.8.3 Antidepressant Treatment Influences Other Neurotrophic Factor Systems

The function and survival of neurons in the adult brain are influenced by a number of neurotrophic and growth factor systems in addition to BDNF. The neurotrophic hypothesis of depression has been extended by studies of some of these factors (see Table 19.3). Stress is reported to downregulate the expression of VEGF in the hippocampus, and this factor is increased by ECS [119, 120]. We have also found that VEGF expression is increased by chemical antidepressants and that VEGF produces antidepressant effects in behavioral models (J. Warner-Schmidt and R. S. Duman, Yale University School of Medicine, 2006, unpublished data).

Another family of factors that has been implicated in depression is FGF. Post-mortem microarray analysis has reported altered expression of FGF and FGF receptors in depressed patients [66]. Preclinical studies demonstrate that chronic antidepressant treatment, including 5-HT and NE selective reuptake inhibitors, increases the expression of FGF2 in the hippocampus and entorhinal cortex [121]. In

TABLE 19.3 Regulation of Neurotrophic/Growth Factors by Stress and Antidepressant Treatment and Influence of These Factors on Behavioral Models of Depression and Adult Neurogenesis

Factor	Stress	Antidepressant Treatment	Behavior	Neurogenesis
BDNF	Decrease	Increase	Antidepressant	Increase
VEGF	Decrease	Increase	Antidepressant	Increase
FGF2	NA	Increase	NA	Increase
IGF-1	NA	NA	Antidepressant	Increase
Activin	NA	Increased	Antidepressant	NA

Note: The influence of stress (column 2) or antidepressant treatment (column 3) on the expression of each neurotrophic/growth factor is indicated (decrease or increase). The influence of neurotrophic/growth factors on behavioral models of depression or adult neurogenesis is indicated in the last two columns. Antidepressant indicates that the factor produces an antidepressant-like response. NA, not analyzed

addition, the coadministration of an SSRI with an atypical antidepressant, a combination that has proven effective for treatment-resistant depressed patients, results in a greater induction of FGF2 than with either treatment alone [122]. The regulation of FGF2 could also play a role in the regulation of adult cell proliferation (see below).

There are a number of other factors that are regulated by antidepressant treatment and that have been implicated in depression, including glial cell–derived neurotrophic factor [123], activin [14], and IGF [124]. Additional studies are necessary to further test the role of these factors in depression and the response to antidepressant treatments. Moreover, the possible interaction of these factors in cellular and behavioral models must be examined.

19.9 INFLUENCE OF ANTIDEPRESSANT TREATMENT ON ADULT NEUROGENESIS

Downregulation of adult neurogenesis by stress and decreased hippocampal volume in stress-related illnesses suggest that alterations of the numbers of neurons and glia contribute to the pathophysiology and treatment of these disorders. Recent studies support this hypothesis and examine the mechanisms underlying the regulation of adult neurogenesis (see Fig. 19.3).

19.9.1 Antidepressant Treatment Increases Adult Neurogenesis

Studies of antidepressants support the hypothesis that the therapeutic effects of these agents may occur at least in part via upregulation of adult neurogenesis (see [2, 38]). Chronic but not acute antidepressant treatment increases the number of newborn cells in the adult hippocampus, and this effect is observed with different chemical classes of antidepressants [125–128]. Antidepressant treatment can reverse or block the effects of stress on adult neurogenesis [44, 129–131]. Adult neurogenesis is also upregulated by other treatments that have antidepressant effects, including exercise (see [38]).

A direct link between neurogenesis and antidepressant response has also been examined. Blockade of antidepressant induction of adult neurogenesis, either by focused irradiation or in 5-HT_{1A} mutant mouse, results in blockade of the behavioral effects in models that are responsive to antidepressant treatment (i.e., novelty suppressed feeding and chronic mild stress) [128]. There is no effect of decreased neurogenesis on basal responding in these tests, indicating that neurogenesis is required for an antidepressant response but that reductions are not sufficient to produce a depressive-like phenotype. This suggests that the effect of stress must include additional neurochemical and cellular effects that are required to produce depression in these animal models. Alternatively, it is possible that the animal models available and the underlying cellular mechanisms that influence behavior do not accurately reflect mood disorders in humans.

19.9.2 Mechanisms Underlying Antidepressant Regulation of Adult Neurogenesis

The investigation of adult neurogenesis is an area of tremendous interest and potential and could lead to the development of pharmacological agents that directly

and specifically control cell proliferation and survival. This information would have multiple applications for neurological disorders as well as psychiatric illnesses such as depression. A significant number of studies have been focused on neurotrophic/growth factors, including several of those implicated in depression (see Table 19.3 and Fig. 19.3). For example, infusions of BDNF into the lateral ventricles increase neurogenesis in adjacent brain regions but not in the hippocampus [49, 50]. A study of BDNF(+/−) mice reports that BDNF is necessary for basal rates of proliferation in the hippocampus, [132] although another study of mutant mice did not find effects on proliferation but rather found evidence that BDNF enhances the survival of newborn neurons in the hippocampus [133].

Studies of VEGF have also been very interesting, as this factor has been demonstrated to increase neurogenesis in the adult hippocampus [51, 52, 134]. Peripheral VEGF is required for the upregulation of adult neurogenesis by exercise [135]. Moreover, recent studies demonstrate that blockade of VEGF signaling blocks the induction of neurogenesis by ECS and fluoxetine administration (J. Warner-Schmidt and R. S. Duman, Yale University School of Medicine, November 2006, unpublished data). Other factors that are known to regulate adult neurogenesis and that could play a role in the actions of antidepressant treatment include FGF2 and IGF1 [136–138]. Cotreatment with both FGF2 and IGF1 is required for maximal stimulation of the proliferation of cultured progenitor cells [139]. It is possible that a similar coordinated interaction of these or other factors may be necessary for antidepressant regulation of neurogenesis. Additional studies will be required to test this hypothesis and the role of these factors.

19.9.3 Influence of Antidepressant Treatment on Glial Proliferation

Glia make up a significant percentage of the cells in the brain and provide metabolic and structural support for neurons. Stress decreases the number of glia in the prefrontal cortex and a decrease in glia has been reported in postmortem tissue from depressed patients. In contrast, chronic antidepressant treatment increases the proliferation of glia in the prefrontal cortex. This includes ECS and fluoxetine as well as the atypical antipsychotic olanzapine [140, 141]. Newborn glia in the prefrontal cortex express markers of oligodendrocytes and endothelial cells, as well as an unidentified population of cells, but not astrocytes. Endothelial proliferation is also increased by ECS in the hippocampus and amygdala [142, 143].

These findings suggest that antidepressant treatment could reverse the effects of stress and depression on glial proliferation in the prefrontal cortex as well as neurogenesis in the hippocampus. The role of neurotrophic/growth factors in the regulation of glial proliferation remains to be determined.

19.9.4 BDNF Increases Sprouting of 5-HT Neurons

Another interesting effect of BDNF that is relevant to the actions of antidepressants and depression is the regulation of the 5-HT neurotransmitter system. This was first demonstrated by studies showing that infusions of BDNF have a significant effect on the sprouting of either 5-HT neurons, either intact or neurotoxin lesioned [144, 145]. BDNF resulted in increased sprouting of 5-HT axons when infused into either the cerebral cortex or the hippocampus. These findings demonstrate that BDNF can influence growth of intact 5-HT neurons and the survival and recovery of damaged

5-HT neurons. Together with studies demonstrating antidepressant regulation of BDNF, these results demonstrate a reciprocal interaction between the BDNF and 5-HT that could be necessary for the normal function of neurotrophic factor and neurotransmitter signaling in the brain. It is possible that alterations of either factor could be sufficient to produce effects on the other and thereby lead to altered neuronal and behavioral consequences. This could explain either a loss of function if BDNF or 5-HT signaling is decreased or increased function when these systems are positively regulated. Dysregulation of BDNF and 5-HT interactions could also account for certain aspects of neuronal dysfunction in aging [146].

19.10 BEHAVIORAL EFFECTS OF NEUROTROPHIC FACTORS

The opposing actions of stress/depression and antidepressant treatment on neurotrophic factor expression provide strong correlative evidence for a role of these factors in the pathophysiology and treatment of mood disorders. To directly test this hypothesis, investigators have utilized a combination of approaches to either increase or block neurotrophic factor levels and signaling in limbic brain structures (see Table 19.3).

19.10.1 Influence of BDNF in Models of Depression

Behavioral studies of BDNF have provided direct evidence that this neurotrophic factor is sufficient and necessary for an antidepressant response. Infusions of recombinant BDNF into the midbrain [147], hippocampus [148], or lateral ventricles [124] produce robust antidepressant effects in the forced-swim test and/or learned helplessness paradigms. Infusion of NT-3 but not NGF into the hippocampus also produces an antidepressant response, possibly due to the activity of NT-3 for TrkB receptors [148].

A requirement for BDNF and TrkB receptors in the actions of antidepressant treatment in the forced-swim test has also been demonstrated using BDNF(+/-) and TrkB dominant-negative mutant mice [149]. Similar effects were observed in inducible/conditional BDNF mutant mice in the forced-swim test [150]. There was no effect of BDNF mutation or blockade on basal behavior in the latter studies or another study in BDNF(+/-) mice [151] suggesting that decreased BDNF signaling may not be sufficient to produce a depressive phenotype. Another study did observe a helpless phenotype in BDNF(+/-) mice, although this finding is complicated by the increased pain sensitivity in these mice [69]. The viability of constitutive BDNF(-/-) mice is significantly reduced, making it difficult to examine complete null mutants.

One possible explanation for these effects is that partial deletion of BDNF (i.e., constitutive +/- or conditional in forebrain regions) is not sufficient to produce a depressive phenotype. It is also possible that reduced BDNF signaling is not sufficient to produce a depressive phenotype but that it is a risk factor for depression. This possibility is supported by studies demonstrating that exposure of BDNF(+/-) mice to stress or blockade of intracellular signaling pathways activated by BDNF results in a depressive phenotype on the forced-swim test [152]. Further studies will be required to test this hypothesis.

19.10.2 Influence of Other Factors in Models of Depression

Investigations of other neurotrophic factors in models of depression and antidepressant treatment have been initiated. The antidepressant effects of activin [14] and VEGF (J. Warner-Schmidt and R. S. Duman, Yale University School of Medicine, November 2006, unpublished data) have been mentioned, and more recent studies have demonstrated effects of IGF-1. Infusions of IGF-1 into the lateral ventricles are reported to have antidepressant effects in the forced-swim test [124] (Table 19.3). These findings provide evidence that additional neurotrophic/growth factors are capable of influencing responses in behavioral models and warrant further investigations.

19.11 SUMMARY AND CONCLUSIONS

The basic research and clinical studies discussed in this review demonstrate that neurotrophic factor expression is altered by stress and depression and that this deficit is reversed by antidepressant treatment. Upregulation of neurotrophic factors could block or reverse the atrophy and cell loss resulting from stress/depression and thereby contribute to the actions of antidepressant treatment. Additional postmortem studies are needed to further characterize the types of morphological changes that underlie the atrophy of limbic brain structures and to identify the cell types that are affected in depressed patients. Identification of the molecular changes that underlie these morphological alterations will also be necessary. Microarray gene chip analysis is already proving useful in addressing this issue as well as in further characterizing the gene expression changes that occur in response to stress and antidepressant treatments in rodent models. These studies will be important to further test the validity of the neurotrophic hypothesis of depression. Moreover, further identification of the molecular mechanisms underlying the actions of stress and antidepressant treatment will be crucial for elucidation of the pathophysiology and treatment of depression and related mood disorders.

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ANTIDEPRESSANT TREATMENT AND HIPPOCAMPAL NEUROGENESIS: MONOAMINE AND STRESS HYPOTHESES OF DEPRESSION CONVERGE

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20.1	Monoamines and Development of Antidepressants	822
20.2	Hippocampus and Depression	823
20.3	Stress and Monoamine Hypotheses of Depression Converge	825
20.4	Hippocampal Neurogenesis and Depression	826
20.5	Stages of Adult Neurogenesis	827
20.6	Neurogenesis Regulation by Stress hormones	829
20.7	Neurogenesis Regulation by Serotonin and Norepinephrine	831
20.8	Behavioral Readouts and Animal Models	833
20.9	Conclusions and Future Directions	833
	Acknowledgments	836
	References	836

Major depressive disorder (MDD) is a debilitating psychiatric illness affecting up to 20% of the U.S. population. Early onset and high prevalence have made MDD a public health concern throughout the world [1]. Depressed mood, inability to experience enjoyment, and neurovegetative symptoms often render affected individuals unable to engage in their social and occupational roles. Human genetic studies revealed that depression has a heritable component with roughly 40–50% of the risk attributable to genetic factors [2]. The remainder of the risk can be attributed to environmental factors such as insult to the developing brain and/or some forms of psychosocial stress [3]. There are multiple highly effective treatments for depression. Psychosocial treatments focus on the perceptions of stress, stress prevention, and coping mechanisms, whereas most effective pharmacological treatments modulate

monoamines in the brain. Numerous research efforts to understand the mechanisms by which psychotherapy and pharmacotherapy treat depression have resulted in the respective “stress” and “monoamine” hypotheses of depression (reviewed in [2]). This chapter will focus on recent findings that stress and monoamine hypotheses of depression converge in their opposing impact on adult hippocampal neurogenesis.

20.1 MONOAMINES AND DEVELOPMENT OF ANTIDEPRESSANTS

Pharmacological treatment of depression was fundamentally altered in the 1950s with two clinical observations. First, treatment with iproniazid, a monoamine oxidase inhibitor commonly used to treat tuberculosis, led to euphoric experiences in tuberculosis patients. The second observation followed discovery efforts in antipsychotic pharmacotherapy. Imipramine, which inhibits the reuptake of synaptic norepinephrine, serotonin, and histamine, was noted to benefit only schizophrenic patients who were also depressed. These serendipitous clinical observations led to the development of the “monoamine hypothesis” of major depressive disorder [4–7]. Almost 50 years later, selective and nonselective inhibitors of norepinephrine (NE) and serotonin (5-HT) reuptake form the mainstay of antidepressant pharmacotherapy. Despite the excellent efficacy of serotonin reuptake inhibitors (SRIs) and norepinephrine reuptake inhibitors (NRIs), many patients remain refractory to antidepressant treatment. Moreover, the desired antidepressant effect of SRIs and NRIs is often accompanied by undesirable side effects, such as sexual dysfunction, gastrointestinal discomfort, and weight gain. Also, all SRIs and NRIs share a delay in onset of action, which can last several weeks to months. Greater target specificity thus became the strategy to decrease these undesirable properties of antidepressant medications.

Modulation of specific monoamine receptor subtypes took center stage among newer antidepressant strategies. For instance, activating the inhibitory 5-HT_{1A} autoreceptors and inhibiting the stimulatory 5-HT₂ receptors was explored. These strategies resulted in part based on observations that 5-HT_{1A} receptors are decreased and 5-HT₂ receptors are increased in brains from depressed individuals [8], and several compounds acting at the 5-HT_{1A} or 5-HT₂ receptors have been approved for antidepressant use and may show clinical efficacy. However, these and other approaches for developing 5-HT receptor-specific antidepressant therapies yielded mixed clinical results, with the newer drugs not being used as widely as the older reuptake inhibitors.

One of the clinical strategies for using 5-HT_{1A} antagonists has been to augment SRI treatment by decreasing the latency of antidepressant response. Delayed onset of action is a phenomenon shared by almost all classes of antidepressants and belies a simple deficit model of depression. While antidepressants alter synaptic concentrations of monoamines within minutes to hours following administration, the accepted onset of clinical action for this class of medications is on the order of weeks to months. One proposed hypothesis for the delayed onset of action by SRIs was that stimulation of presynaptic inhibitory 5-HT_{1A} receptors (from SRI-induced synaptic 5-HT) resulted in a decrease in 5-HT release into the synapse [9]. Some argued that the delay in the onset of action occurs because the clinical effect of SRIs is not achieved until a desensitization of 5-HT_{1A} receptors takes place [10]. There is some preliminary clinical evidence suggesting that 5-HT_{1A} receptor-specific compounds may hasten the

onset of SRI action (reviewed in [11]). However, clinical results have been underwhelming and such augmentation strategies have not received broad enthusiasm from the clinical community. Thus, even if 5-HT_{1A} desensitization accounts for some of the mechanism underlying delayed onset of antidepressant action, the strictly biochemical explanations remained unconvincing.

20.2 HIPPOCAMPUS AND DEPRESSION

Another hypothesis for the delayed onset of antidepressant action is that this delay may represent a period of time necessary for plastic structural changes to form and accumulate in the treated brain. Research looking at the possibility that structural changes are necessary for response to antidepressants has focused on the hippocampus.

The hippocampus is a bilateral structure located on the medial surface of the temporal lobes running along the anterior–posterior axis in humans and septotemporal and dorsoventral axes in rodents (Figs. 20.1 and 20.2a). Input to the hippocampus comes largely from the parahippocampal gyrus, which is rich in inputs from most regions of the brain. The information is then processed and transmitted through the dentate gyrus and along a trisynaptic circuit. Hippocampal outputs terminate in both cortical and subcortical structures, including rich projections to the amygdala, anterior cingulate cortex, frontal cortex, and hypothalamus, and to the neocortex via reciprocal fibers running through the parahippocampal structures [12]. Most of the areas mentioned above have also been implicated in major depressive disorder [2].

There have been multiple findings that hippocampal volume is reduced in patients with unipolar depressive disorder (reviewed in [13]). Using volumetric magnetic

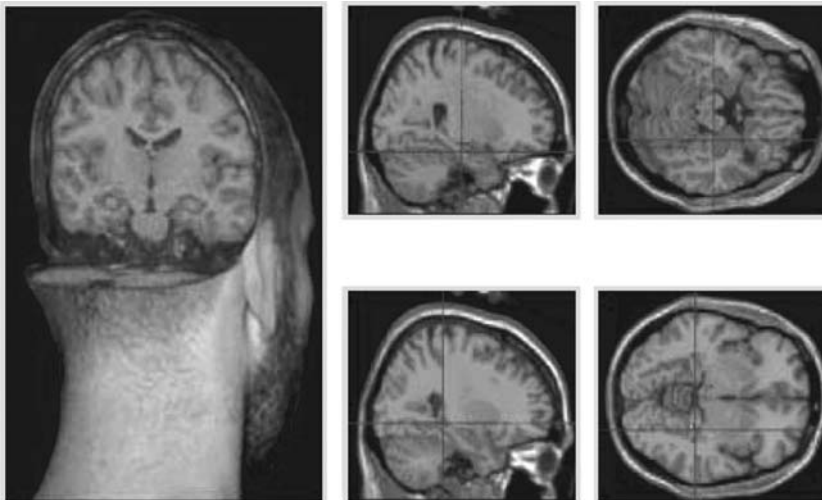


Figure 20.1 Human hippocampus. The left figure indicates the location of the hippocampus in coronal plane within the head. The four figures on the right show functional magnetic resonance imaging (fMRI) of hippocampal activation in sagittal and a transaxial projection images during performance of a novelty detection task. [Reprinted with permission from C. A. Tamminga (2005), *Am. J. Psychiatry*, 162(1), 25.] (See color insert.)

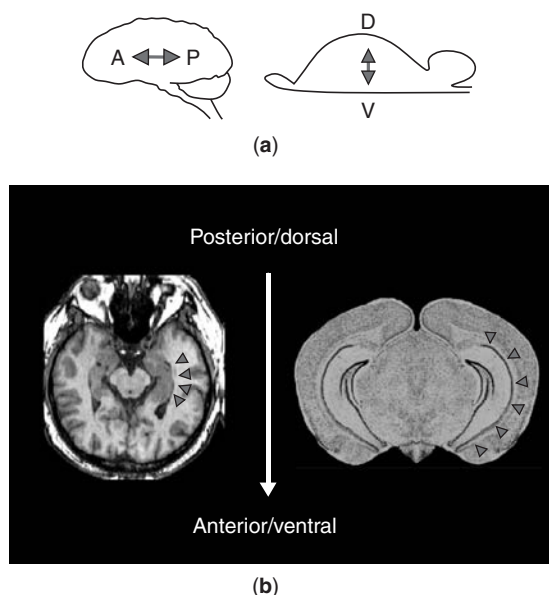


Figure 20.2 Comparative hippocampal anatomy. The dorsoventral axis of the mouse hippocampus (right) corresponds to the anterior–posterior axis in the human brain (left) (a). Transverse MRI image pointing out the hippocampus along the posterior–anterior axis (left). Coronal section through a mouse brain Nissle stained with the hippocampus pointed out along the dorso ventral axis (right) (b). (See color insert.)

resonance imaging (MRI) analyses, several groups have demonstrated that hippocampal volume is inversely proportional to the number of days that individuals were depressed [14–16]. Changes in hippocampal volume also occur in other psychiatric disorders, illustrating the unique plasticity of this limbic structure in response to environmental influences. Some of the documented hippocampal functions include a critical role in short-term memory, depression, and psychosis [17]. Recent studies of hippocampal function have focused on its role as a novelty detector [18] and underscore the role of the hippocampus in the brain's interaction with the external environment.

Perhaps the best-documented relationships between experience and hippocampal volume are the changes in hippocampal volume produced by psychosocial stress. An extensive body of literature documents that adrenal hormones [19] mediate hippocampal sensitivity to psychosocial stress. Neurons throughout the hippocampus express receptors for both glucocorticoids (GRs) and mineralocorticoids (MRs) [20]. Glucocorticoids and mineralocorticoids are released by the adrenal glands in response to environmental stress. In response to chronic stimulation by adrenal hormones, remodeling of the hippocampal neuropil is observed as both decreased structural complexity of CA3 dendritic arbors and a decrease in neurogenesis in the subgranular zone of the dentate gyrus. Concurrently, hippocampal volume decreases. The effect of stress on the hippocampus has been linked to GRs using pharmacological means as well as through studies on transgenic animals. Thus, psychosocial stress results in hippocampal changes, and GRs mediate this phenomenon.

20.3 STRESS AND MONOAMINE HYPOTHESES OF DEPRESSION CONVERGE

Several recent genetic findings provide correlative support for the convergence of stress and monoamines. Caspi and colleagues elegantly demonstrated that a functional polymorphism in the noncoding region of the gene for the 5-HT transporter (5-HTT) conferred increased risk for MDD only in people who suffered multiple stressful life events [21]. Caspi's important observation was recently replicated in another cohort [22]. Conversely, Binder and colleagues reported that a functional polymorphism in a stress-response gene predicted response of depression to monoamine modulators [23]. Chronically elevated cortisol and defective dexamethasone suppression is a reliable finding in some people with MDD [24]. Binder et al. demonstrated that a functional single-nucleotide polymorphism in a gene encoding the glucocorticoid receptor cochaperone FKBP5 predicted a more rapid response to treatment with monoamine-modulating antidepressants. Furthermore, the FKBP5 polymorphism predicted adrenocorticotropin hormone responsivity to corticotropin release hormone challenge following dexamethasone suppression in the depressed subjects. Unfortunately control subjects were not separated by the FKBP5 genotype so it is difficult to say whether or not this polymorphism reflects cortisol regulation in the general population. The 5-HTT polymorphism results suggest that trait 5-HT mediates the effect of psychosocial stress on depression, while the FKBP5 data suggest that trait glucocorticoid responsivity predicts response to serotonergic and noradrenergic medications. Both studies suggest a reciprocal gene-by-environment interaction between stress and monoamines, where the environmental manipulation of one system interacts with the genetic contribution by the other in the manifestation of depressive symptoms. These data provide genetic evidence for the convergence of the monoamine and stress hypotheses of depression.

Evidence suggesting that both psychosocial stress and monoamines affect the hippocampus in animal models of stress and depression and in brains from depressed individuals highlights the above human genetic observations. Genetic and pharmacological manipulation studies suggest that monoamines modulate the effects of stress on the hippocampus and vice versa. The effects of stress on the hippocampus are pharmacologically and genetically reversible. Fluoxetine reverses both the behavioral sequelae and the decrease in cellular proliferation resulting from inescapable shock [25]. Similarly, the monoamine oxidase inhibitor moclobemide reverses the decrease in proliferation resulting from chronic stress [26]. Stress-induced decrease in hippocampal volume reverses with the serotonin reuptake enhancer tianeptine [27]. This drug also reverses stress-induced decreases in cellular proliferation, dendritic arborization, and release of brain-derived neurotrophic factor (BDNF) [27, 28]. Similar hippocampal responses to 5-HT reuptake enhancement or inhibition appear paradoxical. Explanations for these observations include an intriguing possibility that chronic enhancement or inhibition of the 5-HTT may result in similar molecular and ultimately structural and physiological adaptations by the cells. Another possibility that has some experimental support [28a] is that tianeptine exerts its effects on the hippocampus through a non-serotonergic mechanism. In one study, tianeptine was effective in preventing stress-induced decrease in dendritic arborization in CA3, while the SRIs fluoxetine and fluvoxamine were not [28]. This is the only evidence suggesting that stress-induced decrease in cellular

proliferation and dendritic arborization is pharmacologically dissociable. The connection, or lack thereof, between stress-induced decreases in neurogenesis and dendritic arborization in the CA3 remains to be adequately explored.

Human studies on the effect of antidepressant treatment on the hippocampus parallel the results of the animal findings [29]. Patients who suffer from posttraumatic stress disorder (PTSD) demonstrate increased performance on verbal declarative memory tasks and increased hippocampal volume following treatment with the SRI paroxetine. Consistently, gene expression studies in animals suggest the hippocampus as a site of convergence between stress and monoamines. One report indicates that stress-induced changes in hippocampal expression of genes related to proliferation and plasticity were reversed by treatment with the tricyclic antidepressant clomipramine in tree shrews [30]. Of course, 5-HT and NE receptors are expressed throughout the brain, and precise anatomical targets for the clinical effects of antidepressants have not been elucidated. Nevertheless, taken together these findings strongly support a role for 5-HT in modulating stress-induced changes within the hippocampus. Accordingly, investigation into biological mechanisms by which monoamines and stress modulate hippocampal plasticity became of paramount importance in understanding the biological mechanisms of antidepressant action.

20.4 HIPPOCAMPAL NEUROGENESIS AND DEPRESSION

Brain plasticity was traditionally accepted to result from chemical and possibly structural changes in existing neurons. The possibility that neuronal cells are continually added to the adult brain was intensely rejected by the scientific community until very recently [31]. However, the advent of newer techniques to detect DNA replication and thus cell division have led to the acceptance that new neurons are generated in the adult brains of rodents and other mammals. In a seminal study, Erickson and colleagues demonstrated that neurogenesis occurs in adult humans by identifying new neurons, which incorporated the thymidine analog bromodeoxyuridine (BrdU), in the hippocampi of deceased cancer patients who received BrdU as a diagnostic agent [32]. With the eventual acceptance that adult neurogenesis was pertinent to human neurobiology, great interest developed in directing neurogenesis to combat neurodegenerative disease. Sophisticated cell culture models focused on identifying neuronal progenitor cells and elucidating mechanisms of division and differentiation of these progenitors into functioning neurons. To date, there have been no large clinical implantation trials for neuronal progenitors. Moreover, clinical attempts to implant fetal dopamine-producing cells into the substantia nigra to combat Parkinson's disease have not met with success [33]. Limited success in employing neurogenesis for the treatment of neurodegenerative illness necessitated a closer analysis of the normal function of neuronal turnover in the adult brain. Some clues to the normal function of neurogenesis came from an observation that, although robust, adult neurogenesis is exclusively restricted to the layer of granule cells lining the ventricular system (SVZ) and subgranular zone of the dentate gyrus in the hippocampus (SGZ) in rodents (see Chapter 7 in Volume 3 of this handbook). In humans, adult neurogenesis has been conclusively demonstrated only in the SGZ [32].

The normal function of adult neurogenesis, if there is one, must reflect the normal function of the two brain structures to which it is restricted. The hippocampus has

been firmly established in playing a critical role in learning and memory and a role in the brain's response to psychosocial stress, as described above. The possibility that adult neurogenesis contributes to hippocampus-dependent learning and memory is currently under intense investigation [34]. Observations that the hippocampus may play a role in affective and anxiety disorders have stimulated investigations of hippocampal role in rodent models of anxiety and depression. Several lines of evidence suggest that hippocampal neurogenesis is relevant to the pathophysiology of affective and anxiety disorders. Exposure to mental stress reproducibly decreases neurogenesis in rodent models. Social subordination decreases proliferation of adult hippocampal progenitors in marmosets [35], tree shrews [27, 36, 37], and rats [38]. Immobilization [39], physical restraint [40], and foot shock [25, 39] all decrease incorporation of BrdU into dividing hippocampal progenitors in rats, as does chronic mild stress in mice [41]. Treatment with antidepressants—including SRIs, NRIs, monoamine oxidase inhibitors, and electroconvulsive therapy—increases neurogenesis in the hippocampus (reviewed in [42]). Provocatively, antidepressant treatment reverses the effects of chronic unpredictable stress in mice [41] and of inescapable shock in rats [25] on hippocampal neurogenesis. The reversal of stress-induced decrease in hippocampal neurogenesis by antidepressants is reminiscent of the reviewed effect of stress and antidepressant treatment on hippocampal volume and dendritic morphology. Nevertheless, the mechanism by which chronic stress modulates hippocampal neurogenesis in the dentate gyrus and dendritic arborization in CA3 and whether these two events are related remain unsolved beyond an established role for the hypothalamic–pituitary–adrenal (HPA) system in both. Efforts at elucidating the mechanisms by which stress modulates hippocampal changes are ongoing.

Still more intriguing is the role of these two hippocampal adaptations in the behavioral response to chronic treatment with antidepressants. In a provocative study Santarelli and colleagues demonstrated that neurogenesis was necessary for antidepressants to induce behavioral changes [43]. The investigators ablated hippocampal neurogenesis in mice by specifically exposing hippocampi to X-ray irradiation, which interferes with DNA synthesis and results in death of dividing cells. There was no evidence of death of mature hippocampal neurons and SVZ neurogenesis was spared. Control mice show more exploratory behavior in the novelty-suppressed feeding or improved coat state in the chronic unpredictable stress tests in response to chronic treatment with fluoxetine or imipramine. Following ablation of hippocampal neurogenesis, mice failed to show the same responses to antidepressant treatment. A similar effect was not seen when the SVZ neurogenesis was ablated and in sham-irradiated animals. These data are first to bring forth a possibility of a causal relationship between increased neurogenesis and antidepressant action.

20.5 STAGES OF ADULT NEUROGENESIS

Understanding the diverse stages of hippocampal progenitor maturation is necessary to examine the putative cellular targets of antidepressant medications. Within the hippocampus, neurogenesis is restricted to the subgranular zone of the dentate gyrus (Fig. 20.3). (See also Chapter 7 in Volume III of this handbook.) There, radial glia which express markers of differentiated and activated astrocytes undergo asymmetric division into two daughter cells. The daughter that does not inherit the prolonged

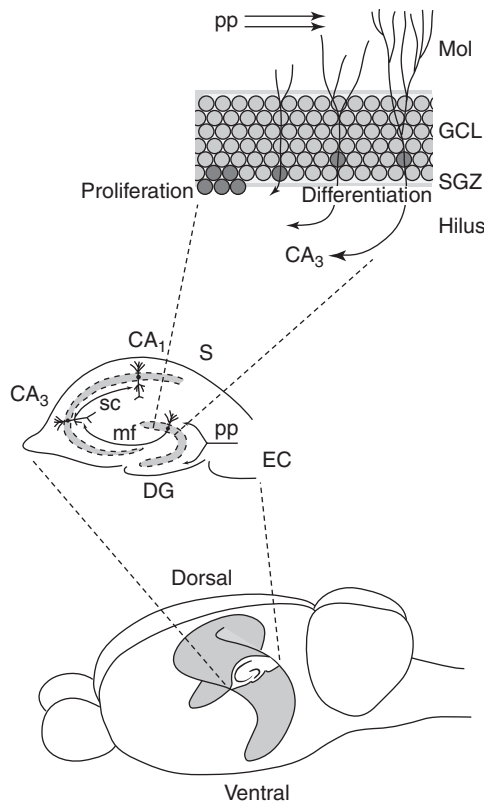


Figure 20.3 Neurogenesis in the adult mouse hippocampus. The hippocampal formation extends longitudinally along a dorsoventral axis in the rodent brain. A transverse section reveals the characteristic trisynaptic circuit that is enlarged in a separate panel. The perforant path (PP) comes from the entorhinal cortex (EC) and innervates the dendrites of granule cells in the molecular layer (Mol) of the dentate gyrus (DG). Granule cells send axons to CA3 pyramidal neurons that constitute the mossy fibers (mf). Schaffer collaterals (sc) are the projections of CA3 pyramidal neurons toward CA1 pyramidal neurons, which in turn project to the subiculum (S). The dentate gyrus is composed of the molecular layer containing the dendrites of the granule cells, the granule cell layer (GCL) containing the cell bodies of the granule cells, and the subgranular zone (SGZ) containing the proliferating progenitor cells. As these progenitors differentiate into mature granule cells, they migrate within the inner third of the GCL and extend progressively longer and more complex dendrites in the molecular layer. [From F. Doetsch and R. Hen, *Curr. Opin. Neurobiol.* 15(1), 121–128. Reprinted with permission from Elsevier.] (See color insert.)

morphology of the radial glia undergoes symmetric divisions in a subgranular pool of cells referred to as the rapidly proliferative pool. At each division, the cell from the proliferative pool undergoes a fate decision of either dividing or migrating into the dentate to differentiate into a granule or a glial cell. Interestingly, the fate-determining decision is dependent on excitatory neuronal activity [44] and is thus regulated by neuronal inputs into the dentate.

Upon migrating into the granule layer, the “immature” neurons express markers that are not expressed by mature granule cells. Specific proteins with established roles

in neuronal migration have a tightly controlled window of expression during granule cell maturation in both developmental and adulthood neurogenesis [45–48]. Immature cells form axonal and dendritic processes within 10 days of division [49]. They then go on to develop functional connections with outputs to the CA3 target pyramidal cells [50]. Full characterization of functional connectivity of the new neurons remains to be carried out. Interestingly, the immature neurons have unique physiological properties and do not share excitation parameters with their mature counterparts [51, 52]. Specifically, CRMP 4/doublecortin positive immature neurons express low-threshold calcium channels and are more susceptible to electrical excitation [46]. Consistently, investigators have found that long-term potentiation (LTP) is easier to elicit in young neurons (four to eight weeks old) compared to old ones [46]. The unusual physiological properties of immature neurons, including a possible excitatory role for γ -aminobutyric acid (GABA) [53], are reminiscent of the excitation parameters exhibited by the immature neurons in the developing brain. Since adult hippocampal neurogenesis is persistent, identification of distinct physiological properties in synaptically connected immature neurons enticed speculation that the combined pool of immature cells may have a unique role in hippocampal information processing and ultimately behavior [34]. In fact, several attempts to identify physiological activity attributable to immature cells led to the identification of a distinct form of LTP in the dentate gyrus which is elicited by weak stimulation of the medial perforant path [46]. Ablation of the subgranular zone neuronal precursors by gamma [46] and X-ray [54] irradiation obliterated this form of LTP three weeks later. Thus, immature dentate neurons express distinct proteins, are more susceptible to plasticity, and constitute a cellular pool with conduction properties that are measurable at a circuit level.

Finally, while it is clear from BrdU pulse-labeling studies that cells that survive for the first month following DNA replication are likely to survive for at least six months to one year [55], it is also clear that many cells undergo apoptosis and die at some point prior to one month. The net cellular turnover in the adult dentate gyrus is difficult to accurately assess using BrdU incorporation, given the dilution of the nucleotide in a rapidly dividing cellular pool. The possibility that older neurons in the dentate gyrus die to make room for newer ones and that this process may facilitate the encoding of experience is especially intriguing and needs to be experimentally assessed. Thus, what is broadly termed *adult hippocampal neurogenesis* involves steps of asymmetric division of glia, rapid division of neuronal progenitors, fate selection, differentiation and migration, maturation, and cellular survival as an overarching theme. Antidepressants and stress may exert their behavioral effects at any of these steps by increasing neurogenesis, influencing the function of young neurons, or directing the integration of new neurons into select circuits.

20.6 NEUROGENESIS REGULATION BY STRESS HORMONES

The effect of psychosocial stress on adult neurogenesis has already been discussed, as was the role of adrenal hormones in mediating stress-induced hippocampal changes. The possibility that the new cells may directly incur the toxic effect of stress on the hippocampus has been investigated in several animal models. Both glucocorticoids and mineralocorticoids have almost immediate transient surges following stress exposure. Early studies demonstrated a sustained increase in cells

undergoing DNA replication in adrenalectomized rats [56]. Later studies conclusively demonstrated that acute and chronic administration of corticosterone decreased proliferation of neuronal progenitors [57, 58]. Administration of a synthetic GR agonist similarly decreased proliferation of hippocampal cells fated to become neurons [59].

While removing glucocorticoid stimulation increases cell proliferation, it is also reproducibly associated with death of mature neurons in the dentate [60] as well as other hippocampal regions [61–63]. Adrenalectomy-associated hippocampal cell death is preceded by GR down regulation and can be reversed by administration of exogenous glucocorticoids [64]. While the neurotoxic effects of adrenalectomy were reversed by glucocorticoid treatment, the proliferative effect was not [57]. Nevertheless, taken together with the observation that GRs and MRs are present on neurons throughout the hippocampus [20], the data suggest that neurogenesis and the survival of mature hippocampal neurons are under adrenal hormone regulation. Moreover, the two effects of adrenal hormones—progenitor proliferation and mature cell survival—appear to be dissociable.

In order to shed light on the mechanisms by which stress regulates neurogenesis, Garcia and colleagues surveyed the expression of GRs and MRs on dentate granule cells at different stages of maturation in adult mice [65]. The investigation revealed that GR is expressed differentially on radial glia and rapidly proliferative cells but not at all on immature neurons. As described by others, GR was expressed on all mature neurons. MR was present only on mature cells, and the proportion of the cells in each pool that expressed MRs differed with the age of the animals. These results suggest that stress can differentially regulate neurogenesis via glucocorticoids directly during the early stages of maturation, while both glucocorticoids and mineralocorticoids can directly impact mature neurons. The role of adrenal hormones in the survival of maturing neurons, if there is one, is likely to be indirect given the absence of GRs or MRs on immature neurons. Thus, differences in GRs and MRs on cells at various points of maturation suggest that psychosocial stress could regulate hippocampal function by selective and different effects on neurons at different stages of maturation.

Stress during certain periods can have lasting effect on neurogenesis and on the response of neurogenesis to stimulation by glucocorticoids. Early, prolonged maternal separation has long-lasting effects of decreasing hippocampal neurogenesis in rats [66]. This effect was reversible by lowering corticosterone levels in the maternally deprived animals when they were adults by adrenalectomy with low-dose corticosterone replacement. A similar reduction of corticosterone did not affect neurogenesis in animals that were not maternally deprived. Interestingly, the HPA axis of the separated animals responded appropriately to stressful stimuli when they were adults, but the effect of early stress on neurogenesis was sustained at all time points tested. Specifically, there was a decrease in cell proliferation and immature neurons but no significant changes in the number of mature neurons. The maternally deprived animals also failed to suppress neurogenesis in response to acute stressful stimuli later in life, notwithstanding their normal HPA response. The baseline corticosterone levels did not differ between deprived and control animals. Together these data suggest that early life stress has long-lasting effects on how hippocampal progenitors respond to glucocorticoids and are reminiscent of the human literature on early life stress conferring lasting risk for depression.

20.7 NEUROGENESIS REGULATION BY SEROTONIN AND NOREPINEPHRINE

Observations that hippocampal neurogenesis was increased in animals treated with antidepressants and that stress-induced decrease in neurogenesis could be reversed by antidepressant treatment sparked much speculation that neurogenesis is regulated by monoamines. Early studies showed that chemical denervation of serotonin fibers by injection of toxins into the dorsal and medial raphe, where all of the serotonin-producing cell bodies reside, reduced hippocampal and subventricular neurogenesis [67]. Full depletion of serotonergic innervation over a one-month period reduced cell proliferation by 60%, and reinnervation of the DG either after prolonged recuperation [68] or following direct implantation of raphe neurons into the DG [69] increased proliferation in the same model. Interestingly, implantation of nonserotonergic embryonic brain tissue into the DG did not have the same effect on cell proliferation, suggesting that serotonin was an important regulatory element. In the same set of studies, investigators also noted that PSA-NCAM (a marker of immature neurons) was affected in a manner consistent with the observed changes in proliferation. Unfortunately, the time course of analysis did not allow the investigators to answer the question of whether immature cells were affected as a consequence of modulating progenitor proliferation or if serotonin affected the immature cells themselves. These studies demonstrated that 5-HT plays a role in progenitor cell proliferation in the DG; attention was subsequently focused on the mechanisms by which serotonin achieves this effect.

Efforts to discern which serotonin receptors could modulate the 5-HT effect on neurogenesis produced several results. Early reports of a possible role for 5-HT_{1A} in cell division in tissue culture models actually precede the papers firmly establishing the presence of neurogenesis in the mammalian brain [70]. Akbari and colleagues noted that the fetal microcephaly induced by treating pregnant rats with cocaine was reversed if the neonates were treated with 5-HT_{1A} agonists [71]. The investigators then argued that the receptor was required for developmental neurogenesis. Further investigation of the role of 5-HT_{1A} in neurogenesis demonstrated that exposure to antagonists of the postsynaptic receptor resulted in decrease in adult hippocampal neurogenesis [72], while treatment with a 5HT_{1A} agonist had the opposite effect [43]. Taken together, the tissue culture studies and the rodent pharmacological manipulations are consistent with each other and point to a positive regulatory role for the 5-HT_{1A} receptors in neurogenesis.

A more recent pharmacological analysis investigated the ability of several 5-HT receptors to regulate neurogenesis [73]. Inhibitory 5-HT_{1A/1B} and stimulatory 5-HT_{2A/2C} receptors have been implicated in depression and anxiety and were examined in the study. The investigators confirmed the earlier observations that activating the 5-HT_{1A} receptors increases proliferation in both the SGZ and the SVZ. Both direct induction of proliferation and rescue of proliferation after serotonergic depletion were assessed. The latter was used as a measure of postsynaptic heteroreceptor activity since depletion acts to mimic maximal effects of the inhibitory autoreceptors. The investigators performed a detailed analysis of proliferative changes in both the SGZ and the SVZ and found notable differences. First, stimulation of 5-HT_{1A} increased progenitor cell proliferation in both the SGZ and SVZ. However, rescue studies suggested that 5-HT_{1B} autoreceptors regulated

neurogenesis in the SVZ and 5-HT_{1B} heteroreceptors regulated neurogenesis in the SGZ. Moreover, stimulation of 5-HT_{1B} autoreceptors decreased SVZ neurogenesis while antagonism at the 5-HT_{1B} autoreceptors increased SVZ neurogenesis. The effect of 5-HT_{1B} on neurogenesis was thus very different from the effect of 5-HT_{1A}. The 5-HT_{1B} results may suggest that 5-HT_{1B} agonists are not ideal candidates for antidepressant development since efficacious antidepressants stimulate neurogenesis in the SGZ but not in the SVZ [43, 74].

Modulation of the 5-HT₂ receptors as targets for antidepressant treatment was discussed in the beginning of the chapter. Several antidepressants (mirtazapine, nefazadone, trazadone) have affinity for the 5-HT_{2A/2C} receptors. Furthermore, atypical antipsychotics which may have some antidepressant activity [75], [76] modulate 5-HT_{2A/2C} binding in the rat brain [77]. At least one of the atypical antipsychotics (olanzapine) was demonstrated to increase neurogenesis in the SGZ with chronic treatment [78]. Pharmacological manipulation of the 5-HT₂ receptors indicated that stimulating 5-HT_{2A} receptors inhibits neurogenesis in the SGZ while 5-HT_{2C} stimulation increases SVZ neurogenesis [73]. The investigators did not note an effect of 5-HT_{2C} in modulating SGZ neurogenesis. This does not appear consistent with the established antidepressant efficacy of 5-HT_{2C} antagonists. However, very recent work demonstrates that inhibition of the 5-HT_{2C} receptor with systemically administered antagonist selectively increases neurogenesis in the ventral hippocampus with the dorsal hippocampus unaffected [79]. Consistently, stimulation of 5-HT_{2C} is anxiogenic only when the agonist is injected in the ventral part of the hippocampus [80]. Indeed, numerous lines of evidence point to segregation of hippocampal function across the dorsoventral axis with the ventral part more important for affect regulation in rodents [81]. The dorsoventral hippocampal axis in rodents corresponds to the anterior–posterior hippocampal axis in humans (Fig. 20.2). Accordingly, the anterior–posterior human hippocampus is differentially affected by depression [82]. It would thus be interesting to reexamine the results from Banasr and colleagues for the possibility that the lack of 5-HT_{2C} effect in the whole SGZ was due to these receptors modulating neurogenesis only in the ventral hippocampus.

Lastly, while only chronic treatment with SRIs results in a proliferative increase, both chronic and acute treatments with the receptor-specific ligands have the same effect on neurogenesis. To this end, the mechanism by which 5-HT modulates neurogenesis is consistent with antidepressant developments discussed in the beginning of the chapter, where receptor sensitization was invoked to explain the delayed onset of SRI action and the mechanism of action of 5-HT_{1A/2A/2C} ligands. Serotonergic modulation of neurogenesis serves as an alternate hypothesis to receptor sensitization in the mechanism of the delayed action of serotonergic antidepressants. Some of the important studies needed to support this hypothesis are discussed at the end of this chapter.

In contrast to 5-HT, little is known about the mechanism by which NE regulates neurogenesis. NRIs are potent antidepressants, and noradrenergic fibers that radiate from the locus ceruleus project heavily to the DG. Like SRIs, chronic NRI treatment increases neurogenesis specifically in the SGZ, and experimental depletion of noradrenergic cells by chemical lesions results in a decrease in neurogenesis [83]. The 67% decrease in neurogenesis caused by depletion of NE in that study is strikingly similar to the 60% decrease from chemical lesions of the raphe cells [67].

Finally, while SRIs do not alter behavior and do not affect neurogenesis in mice devoid of the 5-HT_{1A} receptors, NRIs do both in 5-HT_{1A} knockout mice [43]. Thus, selective contribution of NE to regulation of neurogenesis in the SGZ is comparable to that of 5-HT and is likely to act via a different mechanism.

20.8 BEHAVIORAL READOUTS AND ANIMAL MODELS

The connection between antidepressant treatment and neurogenesis rests on correlative observations that compounds with antidepressant activity promote neurogenesis and at least one report that this increase is necessary for antidepressants to change rodent behavior [43]. Given the heterogeneity of depressive symptoms and the largely subjective nature of this disease, rodent models can only replicate certain aspects of MDD. The extensive body of literature discussing rodent models of depression is beyond the scope of this review and the reader is directed elsewhere [84, 85]. Nonetheless, it is important to note that the different behavioral tasks utilized in rodent studies model particular aspects of the disease. Conflict-based tasks such as the elevated plus maze, open field, and novelty-induced hypophagia bring into conflict the animal's innate drive to explore with anxiety-provoking stimuli and have greater face validity for anxiety disorders [86, 87]. However, these tasks respond to chronic but not acute antidepressant treatment in a way that is similar to MDD and anxiety disorders in humans [85, 87]. Conflict-based tasks may thus serve as better models for anxiety disorders or the highly prevalent comorbid depression with anxiety. The forced-swim and tail suspension tests seem to have adequate pharmacological predictive validity for known antidepressants without response to strictly anxiolytic benzodiazepines [85]. However, these tests respond to treatment acutely. Finally, learned helplessness has excellent face validity as a model for MDD but also responds to acute antidepressant treatment, thus falling short of recapitulating the delay in human response to pharmacological treatment. It is also important to mention that, consistent with the variability of human response to behavioral challenges, inbred rodent lines show great strain variability in all of the behavioral tests reviewed here [85]. The diversity of animal models for the complex disorder of brain function observed in MDD illustrates the importance of validating research findings by utilizing several models in any study and selecting an appropriate experimental strain.

20.9 CONCLUSIONS AND FUTURE DIRECTIONS

This chapter focuses on an apparent mechanism for convergence of the stress and monoamine hypotheses of depression. Major depressive disorder is widely thought to be linked to psychosocial stress, which often precedes its onset and its episodic exacerbations. Psychotherapeutic and psychosocial treatments for MDD focus on stress reduction, coping skills, and changing attitudes towards and perceptions of events that are experienced as stressful. The biological effects of stress on the human brain have been extensively studied and are most profound in the hippocampus. Serotonin and norepinephrine have also been implicated in the biology of MDD through pharmacological, genetic, and biological studies. Interestingly, studies of

risk-conferring genes, which are involved in 5-HT metabolism, suggest that the genetic risk burden of the 5-HT system is modified by stressful life events [21, 22]. Moreover, at least one study implicates a gene involved in the metabolism of glucocorticoids in genetic risk for MDD [23]. The risk-conferring allele in that study also predicts response to treatment with monoamine modulators. (Thus, the genetic burden of serotonin can be modified by stress, while the genetic burden of stress predicts response to modification of monoamine levels.) These data argue that the contributions of stress and monoamines to MDD are linked mechanistically.

Extensive evidence from animal models suggests that regulation of behavior by both monoamines and stress is associated with structural changes in the hippocampus. Consistently, human postmortem, structural, and functional imaging studies implicate the hippocampus in MDD. Within the hippocampus, we reviewed evidence suggesting that the volumetric changes seen in humans are consistent with decreased dendritic arborization in CA3 and CA1. Moreover, stress and monoamines regulate neurogenesis, and treatment with antidepressants protects the hippocampus from stress-induced decline in neurogenesis (Fig. 20.4). Here it is important to mention that given that neurogenesis is restricted to the SGZ of the dentate gyrus and the dentate constitutes a small portion of the hippocampus, it is unlikely that even a large reduction in neurogenesis could directly account for the observed declines in hippocampal volume. Given that dendrites account for a major component of hippocampal volume, decrease in dendritic arborization is more likely to account for volume reductions seen with stress and MDD. Nevertheless, regulation of hippocampal neurogenesis by stress and monoamines has been well documented, and elucidation of underlying mechanisms is ongoing. The specificity of antidepressant treatments for hippocampal but not subventricular neurogenesis is likewise intriguing. Studies exploring hippocampal response to stress and monoamine modulation implicate neurogenesis as a marker for both events. Taken together, these data suggest that stress and monoamines are involved in both the pathophysiology of depression and the regulation of adult hippocampal neurogenesis. Finally, at least one study demonstrates that hippocampal neurogenesis is necessary for behavioral responses to antidepressant treatment.

Several important experiments are needed to support the provocative observations that hippocampal neurogenesis is involved in the regulation of mood. First, while some of the molecular modulators of cellular proliferation have been identified, most of their cellular targets remain unknown. Future studies will need to address which stages of neurogenesis, if any, are directly impacted by glucocorticoids, monoamines, and other modulators. Most studies to date rely on markers of either cell division or immature neurons to assess the molecular mechanisms controlling neurogenesis. More definitive receptor profiling such as that carried out by Garcia and colleagues [65], combined with inducible genetic mouse models, can define cellular targets for the molecules that regulate neurogenesis. Identification of these cellular targets will be important for more refined therapeutic design if the role of neurogenesis in mood regulation proves to be mechanistic.

In order to more firmly establish the role of neurogenesis in MDD and antidepressant treatment, more corroborating evidence is needed. The requirement of hippocampal neurogenesis for the behavioral action of antidepressants will need to be validated using other behavioral tasks and other methods for ablating proliferation of progenitor cells. To this end a genetic system of ablating neurogenesis was

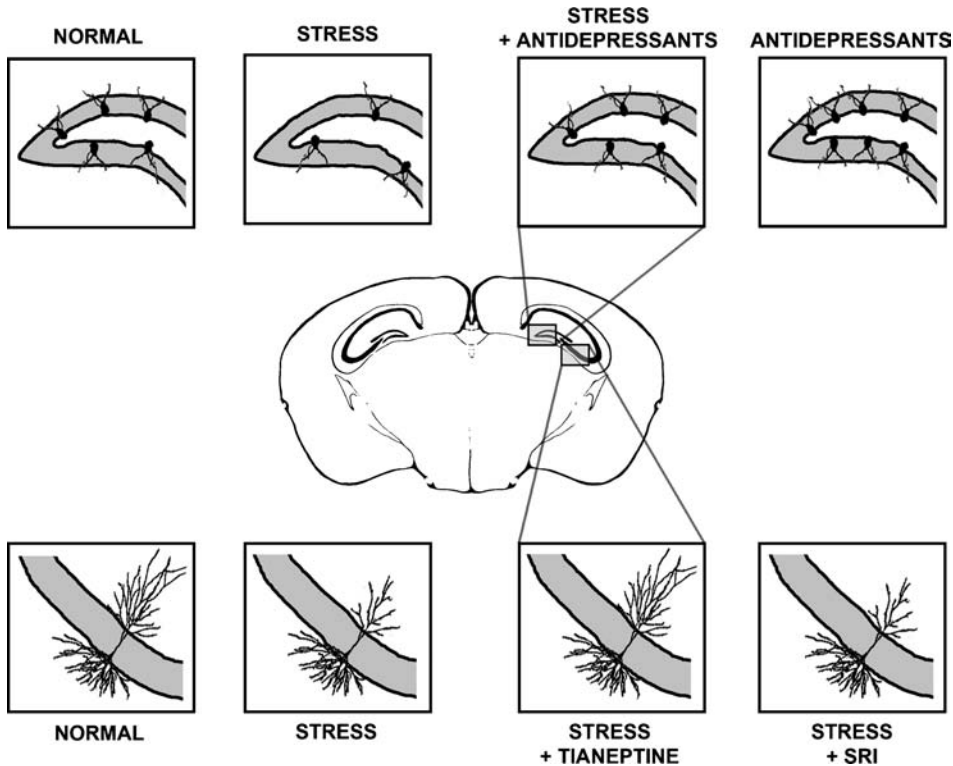


Figure 20.4 Hippocampal neurogenesis, stress, and antidepressant treatment. Schematic representation of a coronal section through a mouse brain. The expanded views in the panels above illustrate the dentate gyrus of the hippocampus with the cell bodies of young neurons in the subgranular layer and dendritic projections traversing the granule cell layer. Baseline neurogenesis is enhanced by antidepressants, reduced by stress, and the stress-induced reduction is reversed with antidepressant treatment. The expanded views in the bottom panels show a pyramidal neuron in CA3 with basal and apical dendrites extending outside the pyramidal cell layer (gray). Stress reduces the baseline arborization of apical dendrites. Tianeptine, but not serotonin reuptake inhibitors, reverses the effect of stress on dendritic arborization [87]. (See color insert.)

recently described [88]. This system is currently being used to repeat the behavioral and physiological results discussed in this chapter [54]. The use of sophisticated genetic manipulations could also allow for exploration of circuit properties of the new cells. While early studies demonstrated that new neurons make functional connections [46, 50, 54], there has been no report definitively characterizing extended circuitry of the new neurons. Delineation of the circuits employed by the new neurons should be useful in ascertaining how antidepressants change behavior at a circuit level.

While many elegant experimental approaches are adding to our insight to the function of neurogenesis in rodents, primate studies on the effect of depression and antidepressant treatment on neurogenesis continue to be lacking. Experimental studies in nonhuman primates should reflect the rodent findings if these data are relevant to human disease. Postmortem human studies are also necessary to help

determine whether stress and perhaps untreated depression decrease neurogenesis while antidepressant treatments increase neurogenesis. Human studies will also help to shed light on whether antidepressants increase hippocampal neurogenesis itself or alternatively prevent the stress-induced decrease in neurogenesis or both, as they do in rodents. Ultimately, naturalistic examples of ablating neurogenesis in humans, such as whole-brain irradiation for cancer treatment, may help to elucidate if neurogenesis is necessary for antidepressants to treat depression and anxiety.

Lastly, it is important to point out that other mechanisms underlying the pathophysiology of depression are likely to emerge. The medications reviewed in this chapter are efficacious for the treatment of depression and anxiety and act by inhibiting the reuptake of 5-HT and NE. These medications activate neurogenesis and are efficacious antidepressants. Yet, depression is a heterogeneous disorder which is likely to engage other brain regions [2, 89]. Consistent with this notion is the observation that there are patients resistant to treatment with SRIs and NRIs. Investigating the biological mechanisms underlying antidepressant treatments that do not alter neurogenesis should delineate other brain systems that are involved in MDD.

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21

NEUROENDOCRINE ABNORMALITIES IN WOMEN WITH DEPRESSION LINKED TO THE REPRODUCTIVE CYCLE

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21.1	Introduction	844
21.2	Circadian Neuroendocrine Studies of Depression During Menstrual Cycle	844
21.2.1	Melatonin	844
21.2.2	Cortisol	845
21.2.3	Thyroid-Stimulating Hormone	845
21.2.4	Prolactin	846
21.3	Circadian Neuroendocrine Studies of Depression During Pregnancy	846
21.3.1	Melatonin	846
21.3.2	Cortisol	847
21.3.3	Thyroid-Stimulating Hormone	847
21.3.4	Prolactin	847
21.4	Circadian Neuroendocrine Studies of Postpartum Depression	848
21.4.1	Melatonin	848
21.4.2	Cortisol	848
21.4.3	Thyroid-Stimulating Hormone	848
21.4.4	Prolactin	848
21.4.5	Estradiol	849
21.5	Circadian Neuroendocrine Studies of Menopause	849
21.5.1	Melatonin	849
21.5.2	Cortisol	850
21.5.3	Thyroid-Stimulating Hormone	850
21.5.4	Prolactin	850
21.5.5	Follicle-Stimulating Hormone	851
21.6	Comparisons of Circadian Neuroendocrine Hormones Across Reproductive Events	851
21.6.1	Menstrual Cycle	851
21.6.2	Pregnancy	851
21.6.3	Postpartum	851
21.6.4	Menopause	851

21.7 Summary	852
21.8 Conclusions	853
Acknowledgments	853
References	854

21.1 INTRODUCTION

Women have at least twice the risk of developing a major depressive episode compared with men. They are prone to develop depressive episodes during times of reproductive hormonal change during the menstrual cycle, pregnancy and the postpartum period, and the menopause transition. Studies in which only single samples of a specific hormone are measured generally have not been revealing with regard to the causes of this depressive diathesis. In contrast, studies in which investigators examine the abnormalities in the circadian rhythms of melatonin, cortisol, prolactin, and thyroid-stimulating hormone (TSH) during these reproductive epochs are more revealing about differences that distinguish depressed patients (DPs) from healthy normal control (NC) women. In this chapter, the authors focus on reviewing studies that measure the circadian profile of hormonal changes in relation to baseline changes in the reproductive hormones of estradiol, progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), comparing DPs with NCs during the menstrual cycle, pregnancy, postpartum, and menopause and across each of these reproductive epochs. Elevated prolactin levels characterize DPs at each reproductive epoch. Increased melatonin levels characterize menopausal and postpartum DPs, whereas decreased melatonin levels characterize premenstrual dysphoric disorder (PMDD) and DPs during pregnancy. Decreased cortisol levels distinguish menopausal DPs from healthy controls. Using circadian measures in well-defined diagnostic groups, further work is needed in order to identify common versus distinctive neuroendocrine profiles for DPs during each reproductive epoch. This information then could serve as a basis for developing more specific treatment modalities.

21.2 CIRCADIAN NEUROENDOCRINE STUDIES OF DEPRESSION DURING MENSTRUAL CYCLE

The depressive disorder linked to the menstrual cycle, PMDD, is defined in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) [1]. Studies in which the investigators examined the circadian rhythms of melatonin, cortisol, TSH, and prolactin in PMDD and NC women during the menstrual cycle are described below.

21.2.1 Melatonin

In healthy women, Nair et al. [2] found a phase delay of the nocturnal peak of melatonin secretion during the midmenstrual period (midcycle), although melatonin was sampled only every 2–4 h at two points in the cycle and without documentation

of estradiol or progesterone levels. Brzezinski et al. [3] and Berga and Yen [4] found that in NC women melatonin circadian rhythms are relatively stable and resistant to hormonal influences during the menstrual cycle. Shibui et al. [5] studied eight healthy women who underwent an ultrashort sleep–wake cycle schedule, a design that uses multiple nap tests to exclude masking effects and determine diurnal fluctuations of the circadian pacemaker. By this method they found that serum melatonin secretion was significantly decreased in the luteal compared with the follicular menstrual cycle phase.

In studies of women with depression, Parry et al. [6] observed that eight women with PMDD had an earlier (phase-advanced) offset of melatonin secretion, which contributed to a shorter secretion duration and a decreased area under the curve (AUC). Melatonin rhythms in eight NC women did not change significantly during four phases of the menstrual cycle in this study. In a larger follow-up study [7] of 21 PMDD and 11 NC subjects, in PMDD subjects, during the symptomatic luteal compared with the asymptomatic follicular menstrual cycle phase, melatonin onset time was delayed, duration was compressed, and AUC, amplitude, and mean levels were decreased. As in the initial study, in NC subjects, melatonin rhythms did not change significantly with the menstrual cycle.

21.2.2 Cortisol

Using the ultrashort sleep–wake cycle design in eight healthy women, Shibui et al. [5] observed that the amplitude of cortisol rhythms was significantly decreased in the luteal compared with the follicular menstrual cycle phase.

In a pilot study of eight women with prospectively documented PMDD who had multiple samplings for cortisol during midcycle (late follicular) and the late luteal menstrual cycle phase, Parry et al. [8] reported increased serum cortisol levels during the midcycle phase. In a study of 20 women with late luteal phase dysphoric disorder (LLPDD) [9] and 11 NC subjects in whom cortisol levels were measured every 30 min from hour 18:00 to 9:00, during the midfollicular (MF) and late luteal (LL) menstrual cycle phases, Parry et al. [10] found that in NC but not LLPDD subjects the cortisol peak was significantly delayed in the LL compared with the MF menstrual cycle phase. In a separate study of 15 women with PMDD and 15 NC subjects, Parry et al. [11] again observed altered timing but not quantitative measures of cortisol secretion in PMDD versus NCs: In the LL versus MF phase, the cortisol acrophase occurred 1 h earlier, on average, in NC but not in PMDD subjects.

21.2.3 Thyroid-Stimulating Hormone

In a study of eight healthy women undergoing ultrashort sleep–wake cycle schedules, Shibui et al. [5] found that the amplitude of the TSH rhythm was significantly decreased in the luteal compared with the follicular menstrual cycle phase.

In a study of the circadian rhythms of TSH in 23 PMDD and 18 NC subjects in which samples for TSH were measured every 30 min from hour 18:00 to 9:00 during MF and LL menstrual cycle phases, Parry et al. [12] observed that TSH rhythms occurred earlier in PMDD than in NC subjects.

21.2.4 Prolactin

In a study of 20 women with LLPDD and 11 NC subjects, Parry et al. [10] measured prolactin every 30 min from hour 18:00 to 9:00 during MF and LL menstrual cycle phases. In LLPDD patients, prolactin peak and amplitude were higher and acrophase earlier than in NC subjects. In a separate study of 23 PMDD and 18 NC subjects, Parry et al. [12] measured prolactin every 30 min from hour 18:00 to 9:00 during MF and LL menstrual cycle phases and found that PMDD patients had higher prolactin concentrations, consistent with previous findings.

21.3 CIRCADIAN NEUROENDOCRINE STUDIES OF DEPRESSION DURING PREGNANCY

Melatonin, cortisol, thyroid, prolactin, and reproductive hormones have been linked to mood changes occurring during pregnancy and postpartum (see reviews in [13–15]), but the circadian rhythms of these hormones have not been the focus of investigation. The studies in which investigators examined the circadian profile of these hormones are described below.

21.3.1 Melatonin

Pang et al. [16] examined plasma levels of immunoreactive melatonin, estradiol, progesterone, FSH, and β -human chorionic gonadotropin (β hCG) during pregnancy and shortly after parturition in 105 Chinese females. In pregnant females, there were significant negative correlations between melatonin and estradiol, melatonin and progesterone, β hCG and progesterone, and β hCG and estradiol and positive correlations between melatonin and FSH and progesterone and estradiol. The findings suggest that gonadal steroids inhibit and FSH potentiates circulating melatonin levels in gravid women and that circulating melatonin in the mother may affect in utero development and be the major source of blood melatonin in the fetus before parturition. Kivela [17] studied 12 women in early pregnancy and 11 women in late pregnancy and found that serum melatonin levels sampled every 4 h during the third trimester of pregnancy were significantly higher than those sampled during the first and the second trimesters and those of non pregnant control women. There was a positive correlation between the week of gestation and serum melatonin concentration at hour 11:00. The amplitude and duration of the nocturnal rise of melatonin were higher during late pregnancy, but there was no clear phase shift. Suzuki et al. [18] examined pregnant women and compared melatonin secretion rhythms sampled hourly from hour 18:00 to 8:00 in six good sleepers and six poor sleepers. Significant differences, using polynomial curve-fitting techniques, were found between the poor sleeper group (lower values) and the good sleeper group (higher values). Non significant trends were found for increased amplitude in the melatonin rhythm in poor sleepers. They also reported significant differences in the cortisol–melatonin ratio between poor sleepers (lower values) and good sleepers (higher values) during late pregnancy as assessed by subjective sleep logs [18]. The differences may reflect changes in the circadian pacemaker system of poor sleepers,

with increases in melatonin release being a response to counteract poor sleep. Nakamura et al. [19], sampling at hours 14:00 and 2:00 in 79 pregnant women (timing during pregnancy not specified), reported increases in maternal serum melatonin until the end of pregnancy which decreased to nonpregnant levels by the second day postpartum. Parry et al. extended initial observations [13] and studied eight women with a major depressive episode (MDE) during pregnancy and four NC subjects matched for age (within five years) and weeks pregnant (within four weeks) in which plasma melatonin was measured every 30 min from hour 18:00 to 11:00 in dim (<50 lux) and dark conditions. As in PMDD patients, DPs had earlier mean melatonin offset time ($p = 0.047$) and lower peak ($p = 0.019$) and AUC ($p = 0.007$) than NC subjects; onset times and durations did not differ ($p > 0.05$). In contrast, other investigators have observed increased melatonin levels or delayed offset in patients with an MDE, mostly in menopausal women [20–25].

21.3.2 Cortisol

Eriksson et al. [26] studied two women in early pregnancy (11–17 weeks), four in late pregnancy (34–38 weeks), and two nonpregnant control women and reported increased cortisol concentrations sampled at 30-min intervals (circadian time epoch not specified) in pregnant women with maintenance of the diurnal rhythm such that nadir levels occurred around midnight and marked elevations during the early morning hours. In the study by Suzuki et al. [18], nonsignificant trends were found for decreased amplitude in the cortisol rhythm. The decreased amplitude of the cortisol rhythm in poor sleepers was due to a suppression of the early morning rise (from 5:00 to 8:00). In an update of studies previously reported by Meliska et al. [27], the ratio of cortisol to melatonin in the morning from 4:00 to 11:00 was higher in DP than in NC subjects ($p = 0.005$).

21.3.3 Thyroid-Stimulating Hormone

Eriksson et al. [26] observed maintenance of a diurnal variation in TSH during pregnancy with maximal values around midnight as in nonpregnant women. An update of Parry et al. [13] found a nonsignificant trend toward lower mean TSH levels in eight pregnant DP as compared with four NC subjects.

21.3.4 Prolactin

Boyar et al. [28] found that in three pregnant women episodic prolactin secretion measured at 20-min intervals for 24 h at 12, 20, and 32 weeks gestation became augmented during nocturnal sleep, with increased mean prolactin levels achieved by increased secretion per secretory episode. Eriksson et al. [26] also reported episodic secretion of prolactin during pregnancy. In the study by Suzuki et al. [18], prolactin levels were high and showed no rhythmicity in both groups of pregnant good sleepers and poor sleepers. In an update of the study by Parry et al. [13], higher prolactin levels were found in eight DP compared with four NC subjects when weeks pregnant and body mass index (BMI) were applied as covariates in the analyses.

21.4 CIRCADIAN NEUROENDOCRINE STUDIES OF POSTPARTUM DEPRESSION

Postpartum depression is classified as an onset specifier for mood disorders in DSM-IV [1]. Studies in which investigators examined the circadian profile of melatonin, cortisol, TSH, and prolactin in relation to reproductive hormones are reviewed below.

21.4.1 Melatonin

Parry et al. [14] measured plasma melatonin every 30 min from hour 18:00 to 11:00 in dim (<50 lux) and dark light in 11 women with an MDE postpartum and 5 NC women approximately matched for age and postpartum month. DPs had a trend toward higher mean melatonin peak and AUC compared with NC subjects. When age, weeks postpartum, and BMI were applied as covariates in the analysis, melatonin offset was later in DPs than in NCs (hour 9:23 vs. 7:52, $p = 0.042$, one-tailed). Other timing measures (onset, duration) were not significantly different between groups.

Although not systematically studied in postpartum women, melatonin levels were reported lower in a majority of studies comparing DP with NC subjects [2, 29–37], and we found and replicated lower melatonin circadian rhythms in patients with PMDD and in combined pregnant and postpartum DPs compared with NCs [6, 7, 13, 38, 39].

21.4.2 Cortisol

Harris et al. [40] measured saliva twice daily for cortisol and progesterone from two weeks before delivery to day 35 postpartum in 120 primiparous women. Seven women developed major depression postpartum. Lower levels of evening cortisol, but not progesterone, in the immediate peripartum period were associated with postnatal depression. In a study of 11 postpartum DP and 5 NC subjects in which serum samples for cortisol were obtained every 30 min from hour 18:00 to 11:00, Parry et al. [41] observed no significant differences in cortisol between DP and NC subjects.

21.4.3 Thyroid-Stimulating Hormone

In a study of 11 postpartum DP and 5 NC subjects in which serum samples for TSH were obtained every 30 min from hour 18:00 to 11:00, Parry et al. [41] found a non significant trend toward lower TSH in DP compared with NC subjects.

21.4.4 Prolactin

In healthy postpartum women, in a rigorous endocrine study using frequent blood sampling, Liu and Park [42] examined prolactin concentrations obtained at 20-min intervals for 12–24 h in eight nonlactating postpartum women on a weekly basis between postpartum days 10 and 26. Serum prolactin levels remained elevated during this time with the diurnal pattern of secretion conserved. With each successive week

postpartum, serum prolactin concentrations declined. Asher et al. [43] examined plasma prolactin levels between hours 8:00 and 9:00 just prior to delivery and three days after delivery in 25 healthy postpartum women. The rise in prolactin plasma levels postpartum had a negative and significant correlation with scores on the Hamilton anxiety scale. The findings suggested that high prolactin plasma levels associated with milk production led to lower anxiety levels in lactating women.

In postpartum DPs, Harris et al. [44] observed that in 147 postpartum mothers, 14.9% of whom were depressed, plasma prolactin levels were inappropriately low in depressed women who breast fed. In other studies, however, hyperprolactinemia has been associated with depressive symptoms [45–47]. In a study of 11 postpartum DP and 5 NC subjects in which serum samples for prolactin were obtained every 30 min from hour 18:00 to 11:00, based on updated data from Parry et al. [41] from breastfeeding mothers, prolactin from hour 4:00 to 11:00 was elevated in DPs relative to NCs [mean (\pm standard error, SE) = 80.1 (\pm 5.2) versus 38.8 (\pm 6.0) ng/mL, p = 0.006] when weeks postpartum and BMI were applied as covariates in the analyses. In contrast, Abou-Saleh et al. [48], in a study in which only a single morning sample was obtained, found significantly lower plasma prolactin levels in women with postpartum depression.

21.4.5 Estradiol

In an update of Parry et al. [41], estradiol was lower in DPs than in NCs (p = 0.035) when age, weeks postpartum, BMI, and breastfeeding status were applied as covariates in the analyses.

21.5 CIRCADIAN NEUROENDOCRINE STUDIES OF MENOPAUSE

21.5.1 Melatonin

In healthy menopausal women, Okatani et al. [49] studied nocturnal serum melatonin concentrations every 2 h in 44 postmenopausal women. They found a steep, age-related decline in nocturnal melatonin secretion lasting up to 15 years postmenopause, with a gradual decline thereafter. Daily oral administration of conjugated estrogen (0.625 mg) suppressed nocturnal melatonin secretion. Based on comparisons with premenopausal women, the authors suggested that transient elevated nocturnal melatonin secretion during menopause might be related to a low-estrogen environment.

In depressed menopausal women, Blaicher et al. [21] evaluated overnight urinary excretion of 6-sulfatoxymelatonin (6-SMT) using a radioimmunoassay in 60 postmenopausal women. Compared with healthy controls, 6-SMT values were significantly higher in depressed females. Patients with hyperprolactinemia showed a trend toward an elevated average nocturnal melatonin concentration. Melatonin levels were significantly lower in patients with insomnia and obesity. In a study of 382 postmenopausal women Tuunainen et al. [23] found that women with major depression compared with NC women without mood disorder, had significantly delayed urinary 6-SMT offset with a trend for increased excretion, particularly in those women with a lifetime history of mood disorders. Kripke et al. [24] found, in 72

volunteers aged 60–78 years, that a lifetime history of any affective disorder was significantly associated with greater urinary 6-SMT excretion in home and laboratory conditions. In an update of a report on the effects of hormone replacement therapy (HRT) in menopausal DPs versus NCs, Parry et al. [25] measured plasma melatonin every 30 min from hour 18:00 to 10:00 in dim (<30 lux) and dark conditions and found, by multivariate analysis, that mean plasma melatonin across all time intervals was higher in 16 DPs than in 9 NCs ($p=0.033$). DPs did not differ significantly from NC subjects on melatonin variables of onset, offset, duration, AUC, or peak at baseline or after 17- β estradiol (E2) treatment.

21.5.2 Cortisol

In a study of 42 healthy menopausal women (mean age 69.6 years), of whom 20 were on estrogen replacement therapy (ERT), Prinz et al. [50] found that elevated 24-h urinary free cortisol was associated with impaired sleep and earlier awakening in older women not on ERT but not in women on ERT.

Antonićević et al. [51] reported that nocturnal cortisol secretion in depressed menopausal women sampled every 30 min between the hours 20:00 and 22:00 and every 20 min between the hours 22:00 and 07:00 was increased in nine postmenopausal patients with depression, while a decrease was noted in nine postmenopausal controls. Parry et al. [25] studied menopausal DPs versus NCs after HRT. DPs had lower serum cortisol levels across time than NCs (hours 18:00 to 10:00, $p=0.012$). Estradiol (oral E2, 1–2 mg) or other treatments [progesterone (P4): medroxyprogesterone acetate, 2.5–5.0 mg in NCs; antidepressant (AD): fluoxetine, 10–40 mg in DP] did not significantly alter mean total cortisol levels in either group. After estrogen treatment, cortisol amplitude ($p=0.016$) and mesor ($p=0.028$) were lower in DPs than in NCs. After estrogen plus progesterone treatment in NC subjects, cortisol peak time occurred earlier compared with baseline ($p=0.036$).

21.5.3 Thyroid-Stimulating Hormone

Ballinger et al. [52] reported that clinically depressed late premenopausal women had significantly higher levels of TSH. In a study of the effects of HRT in menopausal DPs versus NCs by Parry et al. [25], mean TSH levels did not differ between NCs and DPs at baseline. After E2, but not other treatments, TSH values increased relative to baseline in NCs but not in DPs. For TSH nadir, there was a significant treatment–diagnosis interaction. TSH nadir and mesor increased with E2 treatment in NCs but not in DPs.

21.5.4 Prolactin

In healthy menopausal women, Schiff et al. [53] studied the effects of conjugated estrogens (0.625 mg) and placebo in 10 postmenopausal women during sleep and found that estrogen compared with placebo administration blunted LH and prolactin changes but not the rise in cortisol. Fernandez et al. [54] found that morning levels of serum prolactin were lower during menopause compared with pre- or perimenopause. In a study of the effects of HRT in menopausal DPs versus NCs by Parry et al. [25], mean prolactin levels did not differ in NCs and DPs at baseline.

When serum P4 level was included as a covariate in the analysis, adjusted mean prolactin was lower in DPs, overall, than in NCs ($p = 0.020$). E2 treatment produced a treatment–time–diagnosis interaction ($p = 0.014$): DPs, but not NC subjects, had elevated mean prolactin levels from hour 18:00 to 23:00 ($p = 0.001$) and from hour 5:00 to 10:00 ($p = 0.005$) but not from hour 23:30 to 4:30 ($p > 0.05$). Prolactin amplitude increased after E2 treatment in both NCs and DPs ($p = 0.046$) and prolactin mesor was higher in DPs than in NCs ($p = 0.018$).

21.5.5 Follicle-Stimulating Hormone

In an update of Parry et al. [25], FSH was higher in DPs than in NCs ($p = 0.045$) when age and BMI were applied as covariates in the analyses.

21.6 COMPARISONS OF CIRCADIAN NEUROENDOCRINE HORMONES ACROSS REPRODUCTIVE EVENTS

21.6.1 Menstrual Cycle

Studies of circadian hormonal profiles (melatonin, cortisol, TSH, prolactin) in DPs versus NCs during the menstrual cycle did not differ significantly from other women during pregnancy, postpartum, or menopause.

21.6.2 Pregnancy

In pregnant women, mean plasma melatonin was higher compared with women studied during the menstrual cycle ($p = 0.001$), postpartum ($p = 0.001$), or menopause ($p = 0.002$). In DP compared with NC women, however, the mean plasma melatonin level was lower in pregnant DPs than in NCs from hour 4:00 to 11:00 ($p = 0.003$; see Fig. 21.1). Mean serum cortisol was elevated in both NC and DP pregnant subjects, relative to the other groups ($p = 0.001$). Pregnant DPs (but not NCs) had elevated mean LH levels relative to PMDD, postpartum, and menopausal DP (all p values less than zero).

21.6.3 Postpartum

In NCs and DPs, MLT offset was earlier ($p = 0.001$) and AUC was smaller ($p = 0.001$) in postpartum than in menstrual subjects when age, weeks postpartum, BMI, and breastfeeding status were applied as covariates in the analyses.

21.6.4 Menopause

In both NC and DP menopausal women, mean total serum TSH from hour 18:00 to 11:00 was elevated relative to menstruating ($p = 0.015$), pregnant ($p = 0.025$), and postpartum ($p = 0.016$) women. When age and BMI were applied as covariates, FSH was higher in menopausal than in pregnant ($p = 0.001$), postpartum ($p = 0.001$) and menstruating women ($p = 0.001$).

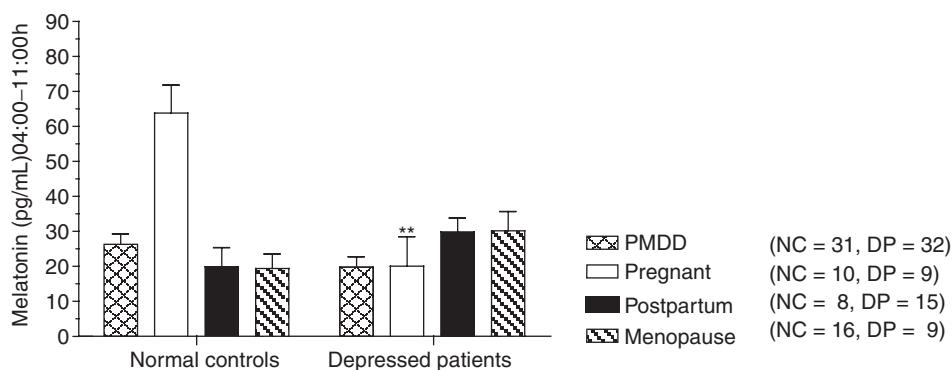


Figure 21.1 Mean morning plasma melatonin levels in NCs vs. DPs. Numerals in parentheses indicate *N* per group. Asterisks denote significant differences between DPs and the corresponding NC group: (**) $p < 0.01$.

21.7 SUMMARY

For menstrual cycle studies of serum or plasma melatonin, the more rigorous studies that sampled more frequently, at 30-min intervals, to obtain more precise timing measures, suggest that in NC subjects melatonin circadian rhythms are relatively stable and resistant to hormonal influences during the menstrual cycle. In contrast, women with well-defined mood symptoms during the menstrual cycle have decreased amplitude (quantitative) measures of melatonin which are associated with changes in phase markers in the symptomatic luteal phase. The findings suggest that depressed patients have a decreased amplitude of the circadian pacemaker that impairs their ability to regulate biological rhythms.

With regard to the limited number of studies of cortisol and TSH circadian rhythms during the menstrual cycle in healthy versus depressed women, the available data suggest more group differences in the timing rather than in the quantitative measures of cortisol secretion. In contrast to cortisol and TSH, increased prolactin amplitude measures differentiate DPs versus NCs during the menstrual cycle, although the findings are based on only two studies.

During pregnancy, some of the inconsistencies in the studies of melatonin circadian rhythms may be attributed to different sampling frequencies and assay and analytic methods and variations in the influence of sleep patterns and gonadal steroid levels. In toto, amplitude measures of melatonin tend to increase in the later stages of pregnancy and are more marked than phase changes. Although the studies that examine the circadian rhythms of cortisol, TSH, and prolactin during pregnancy are few in number with small sample sizes and interpretations are limited by the different methodologies employed, the majority of studies suggest that diurnal rhythms are maintained during pregnancy, with more changes observed in amplitude than in phase. Prolactin levels are higher in depressed compared with healthy pregnant women, as they are in other reproductively related depressive disorders, when covariates of weeks pregnant and BMI are controlled for in the analysis.

Postpartum studies of melatonin, cortisol, TSH, and prolactin circadian rhythms also are limited by small sample sizes and lack of frequent hormonal sampling to

assess circadian rhythmicity. Based on the limited available data, melatonin and prolactin tend to be increased in postpartum depressed patients compared with healthy women. In contrast, in healthy postpartum women, higher prolactin levels are associated with decreased anxiety in lactating women. Estradiol levels are lower in DP compared with NC postpartum women, again when age, weeks postpartum, BMI, and breastfeeding status are applied as covariates in the analyses.

In studies of menopausal women, based on the limited data available from plasma and urinary samples of melatonin, menopausal DPs, compared with NCs, tend to have higher melatonin amplitude measures. In contrast, cortisol circadian rhythms are lower in menopausal DPs compared with NCs. TSH and prolactin circadian rhythms do not differ between depressed and healthy menopausal women. Estrogen treatment, however, has differential effects on cortisol, TSH, and prolactin measures in the two groups. FSH levels are higher in DP than in NC menopausal women when age and BMI are applied as covariates in the analyses.

Across reproductive epochs, it is noteworthy that the mean of melatonin and cortisol circadian rhythms is higher in pregnant compared with menstruating, postpartum, or menopausal women, although in depressed pregnant women the levels of melatonin in the morning are lower than in healthy pregnant women. Evening TSH levels are higher in menopausal DPs and NCs compared with other groups.

21.8 CONCLUSIONS

Certain circadian neuroendocrine features distinguish depressed patients from healthy controls across reproductive epochs: Elevated prolactin levels characterize menstruating, pregnant, postpartum, and menopausal depressed groups compared with healthy control women. Cortisol levels are lower in depressed menopausal women compared with healthy groups as they are in patients with atypical depression [55–58]. Melatonin levels are lower in menstruating and pregnant depressed women and higher in postpartum and menopausal depressed women compared with healthy women. More focus on the pathological timing of circadian rhythms in future studies might serve to clarify these neuroendocrine abnormalities. To make more valid comparisons, these variables will need to be examined for the influence of modulating factors such as age, season of the year studied, BMI, menstrual cycle phase, week pregnant or postpartum, menopausal year, and estradiol and progesterone levels in well-defined diagnostic groups. With more precise measurements and larger sample sizes, further work in each of these reproductively related depressive disorders would serve as the basis of developing treatment modalities targeted to each specific population. These endeavors also have the potential to yield valuable information on how changes in reproductive endocrine function affect the brain's regulation of circadian rhythms and mood.

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NEUROBIOLOGY AND PHARMACOTHERAPY OF BIPOLAR DISORDER

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22.1	Genetics	860
22.2	Postmortem Brain Neuroanatomical Findings	860
22.3	Postmortem Neurochemical Findings	861
22.4	In Vivo Neuroimaging	863
22.5	Peripheral Neurochemical Findings	863
22.6	Mechanism of Action of Lithium	864
22.7	Mechanism of Anticonvulsant Antibipolar Compounds	869
22.8	Conclusion	870
	Acknowledgments	870
	References	871

An ideal chapter on the neurobiology of bipolar disorder (BPD) and its pharmacotherapy would include a review of an integrated understanding of BPD at the molecular, biochemical, neurophysiological, and neuroanatomical levels, with a rational discussion of the site of the therapeutic effects resulting from drug intervention. While this level of knowledge remains to be fully elucidated for most of the known diseases in humans, given the particular complexity of the interaction of the physiological, genetic, and environmental factors within the brain governing the expression of BPD, such understanding at this time appears beyond the horizon. For the purpose of this chapter we will describe some of the most compelling on-going research in this field forming the basis of existing theories of BPD neurobiology and mechanisms of action of antibipolar drugs. At the outset we caution against premature closure, since we do not currently know the most relevant pieces of the puzzle and the most heuristic avenues for investigation may have yet to be identified.

22.1 GENETICS

One well-established fact is the existence of genetic influences on the etiology of BPD, as evidenced in twin and adoption studies. The family pattern is not Mendelian in nature with statistical evidence ruling out a single major gene; rather, BPD is likely inherited through a complex interaction of widely shared genes with low levels of expression, similar to diseases such as diabetes and primary hypertension. Unlike the situation in Alzheimer's and Parkinson's diseases, no pedigree has been discovered where a single gene is operating. Even one such pedigree would jumpstart our studies of the biochemical and molecular pathways involved, as with the discovery of amyloid precursor [1] protein mutations and Parkin [2] mutations.

Linkages between BPD and several large chromosomal regions have been reported in more than one study, although none have been replicated in every study [3]. Moreover, many linkages have been reported and it is likely that some of the findings may remain relevant for selected population genetic isolates and others observed by chance. The chromosomal regions are very broad, and no one gene is clearly implicated. Some of these linkages may reflect nonspecific modifier genes, such as a gene for decreased pelvic diameter that could contribute to increased perinatal hypoxia. Perhaps some of the most promising avenues in this field involve the identification of genes that affect response to mood stimulants and circadian rhythms in both humans and animals [4]. Since such genes have a behavioral phenotype, the pathways around them could contain genes that are etiological and may serve as relevant targets for BPD pharmacotherapy.

22.2 POSTMORTEM BRAIN NEUROANATOMICAL FINDINGS

The absence of postmortem pathology once distinguished psychiatric illness from neurological illness. This concept is no longer heuristic. Harrison and Weinberger [5] recently summarized the neuropathology of schizophrenia by saying that the null hypothesis can be rejected, but a replicable specific defect cannot yet be described. BPD postmortem neuropathology could be similarly described.

Recent studies of postmortem brain tissue find prefrontal cortical and limbic microstructural alterations in patients with bipolar illness compared with controls [6]. Gross neuroanatomical examination of postmortem brain from BPD patients does not reveal any visible changes. The widening of cortical sulci and narrowing of gyri reported by some neuroimaging studies in mood disorder patients are observed in very few postmortem specimens from similar populations. Qualitative microscopic examination of postmortem brain from BPD patients does not reveal any visible neuropathological changes such as focal lesions or the plaques and tangles seen in Alzheimer's disease. However, in some cases, microvascular changes in the gray and white matter are noticeable, although this could be due to repeated stress or drug treatment.

Recent application of nonbiased quantitative stereological cytoarchitectonic cell counting and detailed morphometric methods employed on postmortem tissues from patients with mood disorders provide evidence that mood disorders are characterized by specific changes in the number, density, or size of both neurons and glial cells. Although there are quite a few reports, each based on rather small sample sizes, three

groups have published such studies using well-characterized and matched postmortem samples. These investigations have found reductions in glial cell number and density in frontal limbic cortical regions in BPD subjects [6–9]. The alterations in glia are accompanied by more subtle changes in the density and/or size of specific populations of neurons [6, 8, 9]. Specific types of neurons are also reduced in the hippocampal formation [10] and anterior cingulate cortex in BPD [11], whereas glial cells have not been systematically studied in hippocampus. Some studies indicate increases rather than reductions in the neuronal cell number in the hypothalamus [12, 13] and brain stem nuclei [14–16]. The alterations in cell number and density in BPD are likely to be related to the disorder itself and not to the age, postmortem delay, or time of tissue fixation, since statistical analyses conducted in all of the above-mentioned morphometric studies yielded no significant correlation between cell numbers and any of these confounding variables. It cannot be ruled out, however, that some of the cellular alterations in mood disorders are related to prior treatment with mood stabilizers [6]. Interestingly, postmortem anatomic findings indicate that cellular pathology in the dorsolateral prefrontal cortex (DLPFC) in schizophrenia and BPD differs in magnitude, direction, laminar scope, and relative involvement of neuronal and glial cell types [17].

22.3 POSTMORTEM NEUROCHEMICAL FINDINGS

Torrey et al. [18] summarize results obtained by 56 research groups that studied a single set of specimens (15 each with schizophrenia, BPD, major depression without psychosis, and unaffected control subjects) of the Stanley Neuropathology Consortium. One hundred RNA, protein, and other neurochemical markers were assessed in seven neurochemical systems. Quantitative measures of continuous variables for prefrontal, hippocampus, anterior cingulate, superior temporal cortex, or a combination of these were analyzed. Before correcting for multiple comparisons, 23% of the markers were abnormal in one or more regions, with most indicating decreased expression. Markers that reached statistical significant difference of $\geq 20\%$ and were specific for BPD are complexin 1 and 2 and glucocorticoid receptor in the subiculum, SNAP-25 in the stratum pyramidale, dopamine 5 receptor in the CA1 and the dentate, and synaptophysin and GAP43 in the anterior cingulate. The largest percentage of markers was associated with the developmental/synaptic and γ -aminobutyric acid (GABA) systems. Bipolar disorder and schizophrenia had a 65% overlap. All these findings await further replication in other brain banks.

Since lithium has powerful effects on cell signaling, signaling pathways have been studied as a possible neurochemical etiological abnormality in BPD postmortem brain [19–21]. The function of the phosphoinositide second-messenger system has been assessed in occipital, temporal, and frontal cortex obtained postmortem from subjects with BPD and matched control subjects by measuring the hydrolysis of phosphatidylinositol [22]. There was a selective impairment in the G-protein-stimulated phosphatidylinositol hydrolysis in occipital cortex from bipolar patients compared with control subjects, indicative of a possible impairment in G-protein function. The reason for the selective localization of the deficit is not known. While an initial study by Shimon et al. [23] of prefrontal inositol levels in BPD postmortem brain provided evidence for a reduction, this was not replicated [24]. Differences,

however, have been found in G-protein levels. Elevated levels of the long splice variant of the $G_s\alpha$ subunit were reported in frontal, temporal, and occipital cortices [25, 26] and in the thalamus [27] and of the short splice variant in the hippocampus and caudate nucleus of postmortem brain from BPD patients compared with controls. The long and short splice variants differ in their interaction with the β_2 adrenoceptor [28] and in their potency to activate adenylate cyclase [29], so that changes in their relative expression levels in BPD brain may have consequences for cyclic adenosine monophosphate (cAMP) signaling. However, the increase in $G_s\alpha$ protein levels has not been consistent across studies. It was replicated by Friedman and Wang [30], but not by Dowlatshahi and colleagues [31]. The basis for the discrepancies is presently unknown. It could stem from postmortem differences in clinical and demographic characteristics of the cohort studied, such as drug treatments, agonal state, and tissue-handling conditions. More and better controlled studies with larger sample size will be necessary to validate the changes in G-protein levels in BPD brain.

Within the context of G proteins, Rahman et al. [32] found significantly lower cAMP binding in the cytosolic, but not in the particulate, fractions of cerebral cortices, cerebellum, and thalamus of postmortem brain from BPD patients compared with matched controls, implying that protein kinase A (PKA) regulatory subunit abundance is reduced in BPD brain. Chang et al. [33] found higher protein levels of the regulatory and catalytic PKA subunits in the cytosolic fraction of the temporal cortex from postmortem BPD brains compared with matched controls, consistent with observations of significantly higher levels of basal and stimulated PKA activities and lower activation constant for cAMP in the cytosolic fraction of temporal cortex of bipolar patients compared with controls [34]. Because there is extensive cross regulation among different intracellular signaling pathways and an increasing body of evidence implicates abnormalities in other signal transduction systems in mood disorders, it may be that the disturbances found in the cAMP signaling cascade result from dysregulation of one or more of the signal transduction pathways with which it interacts.

Altered intracellular Ca^{2+} homeostasis in BPD [20] has been suggested to be trait dependent [35]. Ca^{2+} homeostasis affects cellular necrosis and apoptosis [36]. Thus, the elevated PKA subunit levels [33] and PKA activity [34] in postmortem BPD brain described above could reflect a neuroprotective response to processes affecting intracellular homeostasis in BPD.

Prefrontal cortex and cerebellar expression of reelin messenger RNA (mRNA), GAD protein and mRNA, and prefrontal cortex reelin-positive cells have been reported to be decreased by 30–50% in patients with schizophrenia and BPD with psychosis but not in those with unipolar depression without psychosis when compared with nonpsychiatric subjects. The results were, apparently, unrelated to neuronal damage, postmortem interval, dose, duration, or presence of antipsychotic medication. The selective downregulation of reelin and GAD67 in prefrontal cortex of patients with BPD who have psychosis was interpreted by the authors to be consistent with the hypothesis that these parameters are vulnerability factors in psychosis [37].

Abnormal expression of molecules critical for glutamatergic signaling in subcortical structures have been suggested to be associated with the pathophysiology of severe psychiatric illnesses including BPD. CGP39653 and MK-801 are analogs that

bind to the glutamate binding site and the open ion channel of the *N*-methyl-D-aspartate (NMDA) receptor, respectively. In subjects with bipolar disorder the density of MK-801 binding in the CA3 and in the pyramidal and the polymorphic layers of the subiculum and the density of CGP39653 binding across the hippocampal formation were found to be decreased [38]. The density of all GAD67 mRNA-containing neurons was decreased by 35% in layer 2 of the anterior cingulate cortex. The numerical density of GAD67-containing neurons that coexpressed NR(2A) [an NMDA receptor subunit] mRNA was 60% decreased in layer 2 in BPD. The expression level of NR(2A) mRNA in GAD67-containing cells was unaltered. It was concluded that the density of GABA interneurons that express the NMDA NR(2A) subunit is decreased in BPD [39]. Decreased striatal expression of transcripts encoding the postsynaptic density proteins PSD-95 and SAP-102 was found in bipolar disorder while no significant changes in the neurofilament (NF) subunit NF-L and in PSD-93 mRNAs were observed, suggesting dysregulation of cortical-subcortical circuitry [40].

The findings reviewed and others not covered in this chapter do not as yet converge into a comprehensive description of the pathophysiology of BPD. Even previous generalizations that postreceptor intracellular signaling abnormalities rather than pre- and postsynaptic receptor functioning are involved in the etiology of BPD [41] seem to have been premature.

22.4 IN VIVO NEUROIMAGING

The advantages of in vivo neuroimaging over postmortem studies include the avoidance of postmortem artifacts, reduction of long-term drug use artifacts, and the possibility of longitudinal studies that examine patients in all three states of the illness, that is, mania, depression, and euthymia, before and after treatment. However, the marked psychomotor changes in mania and depression may well cause secondary nonspecific brain changes. To our knowledge, no studies use control groups of normals told to overexercise as a control for mania or fasting and sleep restriction as a control for depression. For example, a recent paper using functional magnetic resonance imaging (fMRI) found increased amygdala activation in nine manic patients to a specific task in the left amygdala only. Patients were being medicated and the number of subjects was small [42]. Drevets [43] reported a seminal paper using both positron emission tomography (PET) and MRI reporting decreased grey matter and blood flow in the subgenual prefrontal cortex and abnormal responsiveness to emotional stimuli. This finding, which has a priori specificity, awaits replication. While it is disappointing that most of the in vivo neuroimaging findings are not corroborated by postmortem neuroanatomic data, functional imaging may be providing a more dynamic profile of brain regional dysfunction.

22.5 PERIPHERAL NEUROCHEMICAL FINDINGS

Numerous interesting abnormalities have been reported in peripheral tissues, especially blood cells, of BPD patients. None to our knowledge have been replicable by several groups, and few seem well integrated with postmortem findings or in vivo

neuroimaging. For instance, we reported decreased inositol monophosphatase (IMPase) activity in BPD lymphocytes [44] and were able to replicate this in a second sample and show specificity vis-à-vis schizophrenia and unipolar depression [45]. However, we could not find this decrease of IMPase activity in postmortem brain in BPD [45]. Yoon et al. reported abnormal intracellular Ca^{2+} in a subgroup of BPD patients in B-lymphoblast cell lines [46]. This finding awaits replication and could be related to numerous other abnormalities because of the central role of Ca^{2+} in intracellular signaling. Table 22.1 highlights and summarizes selected molecular and neuroanatomical findings in BPD.

22.6 MECHANISM OF ACTION OF LITHIUM

In 1999 the scientific community celebrated 50 years of lithium's use in the treatment of bipolar disorder. *Goodman & Gilman's Pharmacological Basis of Therapeutics* has been reviewing lithium for over 40 years. In the second edition (1958), lithium salts were claimed to have no therapeutic application and no biological function. They were described as a salt substitute in low-sodium diets where they were found to be toxic. The third edition (1965) recorded that lithium exhibits similar behavior to that of sodium in a number of biological systems, and it was first mentioned that lithium was used in the treatment of the manic phase of manic-depressive illness. The fourth edition (1970) noted that controlled clinical studies indicate that lithium is better than placebo in the prevention of manic disorders, but the mechanism whereby lithium has an effect in affective disorder is unknown. It was noted that lithium may decrease norepinephrine levels at critical receptor sites in the central nervous system (CNS). In the seventh edition (1985) the mechanism of action of lithium was still unknown but mention was made of a "little effect on catecholamine-sensitive adenylate cyclase activity," ligand binding to brain-adrenergic receptors, and a role in inhibiting the effect of receptor blocking agents to cause supersensitivity in adenylate cyclase.

In the eighth (1990) and ninth (1996) editions it was declared that the mechanism of action of lithium remains unknown, although this was now accompanied by the suggestion that biological membranes may be involved. The ninth edition (1996) discusses lithium's modification of hormonal responses as mediated by adenylate cyclase or phospholipase C, developing a transmission-modulation hypothesis of lithium's mechanism of action. In the tenth edition (2001), the mechanism of action of lithium as a mood stabilizer still remains unknown, although multiple cellular effects of lithium were characterized and enumerated. This edition also first describes common targets of lithium and valproate.

These findings may present an embarrassment of riches, since it is difficult to know which data, if any, may be related to mood-stabilizing action and which may be epiphenomena. Herein, we review some of the most prominent current work on the mechanism of action of lithium.

Lithium at therapeutic concentrations inhibits glycogen synthase kinase-3 (GSK-3) as does valproate. GSK-3 was found to be inhibited by lithium in two ways [55]. First, lithium directly inhibits the enzyme. This direct inhibitory effect is amplified in vivo by an increase in the inhibitory phosphorylation of an N-terminal serine in GSK-3, enabling low concentrations of lithium to significantly modulate GSK-3. Inhibition of GSK-3 by lithium has multiple effects on cellular functions due to the numerous

TABLE 22.1 Molecular and Neuroanatomical Findings in BPD

Finding	Tissue/Technique	Possible Implication	Reference
Postmortem Brain			
Reduced oligodendrocyte- and myelin-related gene expression	Gene array	Loss of myelin may lead to aberrant circuits controlling thoughts	[48]
Decreased hippocampal NMDA receptor number but not density	In situ radioligand binding	Altered information processing mediated by hippocampal glutamatergic function	[38]
Occipital cortex hypoaactive phosphatidylinositol (PI) signaling	³ H-PI hydrolysis	Decreased occipital cortex PI signal transduction	[22]
Decreased brain inositol	Cortex, gas chromatography	Deficient phosphatidylinositol second messenger signaling	[23]
Reduced glial density (fewer but larger cells) in cortex	Dorsolateral prefrontal cortex layer III; morphometric three-dimensional cell counting	Abnormal neurodevelopment without gliosis	[9]
Decrease in brain reelin	Prefrontal cortex and cerebellum; studied with immunohistochemistry, reverse transcriptase PCR and Western blotting	Brain extracellular matrix abnormality	[37]
Peripheral Tissue			
Decreased ZIP (Z-Pro-proline-insensitive peptidase) and PO (prolyl-oligopeptidase) in lithium-treated patients	Plasma	Altered cell signaling	[49]
Decreased platelet PLC (phospholipase C) and PKC (protein kinase C) activity and expression of some of isozymes; increased MARCKS (myristoylated alanine-rich C-kinase substrate) expression	Fractionation of cytosol and membranes; Western blotting	Deficient second-messenger signaling	[50]

TABLE 22.1 (Continued)

Finding	Tissue/Technique	Possible Implication	Reference
Increased intracellular Ca^{2+} in a subgroup of patients	Lymphoblastoid cell lines	Abnormal intracellular regulation	[46]
Reduced inositol and IMPase	Lymphoblastoid cell lines	Abnormal phosphatidylinositol second-messenger signaling	[51]
Increased platelet PIP_2 (phosphatidylinositol-4,5-bisphosphate)	Two-dimensional thin-layer chromatography	Impaired cell signaling	[52]
Lower <i>IMP42</i> (one of the two genes coding for IMPase) expression	Lymphoblastoid cell lines	Altered calcium signaling	[46]
Imaging			
Decreased gray matter and blood flow in subgenual prefrontal cortex	Magnetic resonance imaging and positron emission tomography in familial bipolar depressives	Abnormal emotional responsiveness to socially significant or provocative stimuli	[43]
Increased vesicular monoamine transporter (VMAT)	Thalamus and ventral midbrain; binding of VMAT2 by positron emission tomography in vivo	Increased serotonin innervation	[53]
Elevated gray matter lactate and γ -aminobutyric acid	Two-dimensional proton echo-planar spectroscopic imaging	A shift in energy redox state glycolysis > oxidative phosphorylation	[54]
Decreased cortical <i>N</i> -acetyl aspartate	Dorsolateral prefrontal cortex magnetic resonance spectroscopy in vivo	Decreased neuronal density or dysfunction	[83]

substrates of GSK-3, any of which may contribute to the mood-stabilizing action of lithium. These include regulation of neural plasticity through changes in cellular architecture and remodeling events, regulation of gene expression through modulation of the activities of transcription factors, and regulation of cellular responses to stress and the ensuing modulation of cell survival. Jope [55] suggests that the manifold influences of GSK-3 on the dynamics of neuronal function provide support for the proposal that GSK-3 is a key target for the therapeutic actions of lithium. GSK-3 β inhibitors are under development as treatments for Alzheimer's disease and diabetes, and those with an appropriate therapeutic index and pharmacokinetics may someday come to clinical trials in BPD.

Lithium has effects on transcriptional and posttranscriptional mechanisms that modulate the expression of a variety of genes possibly involved in aberrant signaling cascades or impaired cellular resilience implicated in the pathophysiological mechanisms underlying BPD [56]. Of particular note, the prophylactic properties of lithium in preventing recurrent episodes of mania and depression may be attributable to its chronic action in regulating the expression of key proteins in brain. The problem remains to distinguish between primary therapeutic properties linked to the action of lithium versus its secondary side-effect profile. New strategies using DNA chIp-chip technology to address this transcriptional activity of chronic lithium on genes encoding proteins such as PACAP, MARCKS, and cyclin-D, promise to help define a lithium-responsive gene network that will provide a foundation for pathway mapping of novel targets for the development of new drugs with more specific and efficacious mood-stabilizing properties [57]. Application of more recent genomic and proteomic approaches not only will offer improved therapeutic drugs but also will provide needed clues to the underlying etiology and pathophysiology of BPD.

Considerable preclinical and clinical evidence suggests that lithium has neuroprotective and neurotrophic effects which may have relevance for the mechanism of mood stabilization by lithium [58]. Lithium has been noted to have neuroprotective effects in cell culture and in animal models of neurodegeneration such as stroke, Huntington's disease, and Alzheimer's disease, possibly through regulation of expression and/or activity of molecules and enzymes involved in neuroplasticity, neuroprotection, and mitochondrial function, including Bcl-2, GSK-3, and the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway. Emerging clinical evidence additionally suggests that lithium may have similar effects in patients; these findings include an increase in *N*-acetyl aspartate (NAA) and gray matter in patients following long-term lithium treatment. The possibility that lithium enhances neurogenesis reflected in increased grey matter volume is intriguing but has not yet gained consensus. Among four papers by Soares's group two report confirmation [59, 60] and another two report negative replication [61, 62]. A recent comprehensive study [63] also failed to find statistically significant difference of grey matter and limbic volume in lithium-treated patients compared to controls. Moreover, neuroprotection seems to be a common property of many psychoactive compounds, including antipsychotics, antidepressants, and even nonpsychotropic compounds such as several antibiotics [64]. Thus neuroprotection by lithium in a variety of model systems may or may not be related to its mode of therapeutic action.

Chronic lithium may exert its therapeutic effects by normalizing aberrant phosphoinositide signaling through its interactions with inositol monophosphatase and the phosphoinositide pathway, downstream alterations in protein kinase

C (PKC) activity, and substrate phosphorylation [64]. McNamara and Lenox [65] have described that chronic, but not acute, lithium treatment at clinically relevant concentrations significantly reduces the expression of MARCKS (myristoylated alanine rich C-kinase substrate), a primary and preferential PKC substrate, and that MARCKS gene expression is regulated by chronic lithium at the transcriptional level through a lithium-responsive element in the MARCKS promoter. The MARCKS protein is located in pre- and postsynaptic terminals where it has been implicated in the regulation of phosphoinositide hydrolysis, filamentous-actin cytoskeletal plasticity, vesicular trafficking, and the release efficacy of several neurotransmitters. MARCKS is necessary for normal mouse brain development and remains highly expressed in several key adult cortical, limbic, and subcortical brain regions. Several neuroplastic events are sensitive to alterations in MARCKS expression, and prolonged elevations in PKC activity, physiological stress, and proinflammatory factors induce alterations in MARCKS expression. The long-term mood-stabilizing effect, as well as some side effects (memory impairment), of chronic lithium may stem from its capacity to down regulate MARCKS expression and/or modulate its phosphorylation in dysregulated circuits.

Lithium inhibition of adenylate cyclase (AC) has been known for several decades but has not developed into a platform for new drug development [66]. Lithium's effect on this system would have been expected to result in widespread undesired effects in multiple tissues. However, the cloning and biochemical characterization of 10 (AC1–AC9 and SAC) nonclustered AC isoforms distributed among different chromosomes revealed that the different isoforms also have distinct regulatory properties [67, 68] so that the characteristics of the response in a given cell depends on the AC isoform(s) present in the cell [69–72]. Within the brain, AC isoforms localize to discrete regions and show differential regulation by Ca and by G-protein subunits; for example, AC1 and AC3 are expressed in hippocampus, and neocortex, stimulated by Ca-calmodulin, and thereby could be activated by NMDA and involved in synaptic plasticity. High AC2 expressions appear in the hippocampus, hypothalamus, and cerebellum and moderate ones in neocortex, piriform cortex, and amygdala. AC4 is often referred to as “striatal adenylyl cyclase.” AC5 seems to be associated uniquely with dopamine innervation. AC5 and AC6 (also enriched in brain) are inhibited by physiological Ca concentrations. AC7 brain levels are low [67]. Future discovery of differing thresholds for lithium inhibition among the isoforms may pave the way for the AC hypothesis to be revisited.

Chuang [73] studied the protective effect of long-term lithium treatment against glutamate-induced, NMDA receptor-mediated, excitotoxicity in primary cultures of rat brain neurons. The neuroprotective effects of lithium are accompanied by a reduction in NMDA receptor-mediated calcium influx, upregulation of the anti-apoptotic mitochondrial protein Bcl-2, downregulation of proapoptotic p53 and Bax, and activation of the cell survival signaling pathway. In addition, lithium antagonizes glutamate-induced activation of c-Jun N-terminal kinase (JNK), p38 kinase, and AP-1-binding and suppresses glutamate-induced loss of phosphorylated cAMP response element binding protein (CREB) due to activation of protein phosphatase-1. In addition, lithium induces BDNF and activates its receptor TrkB in cortical neurons, which may be implicated in the putative neuroprotective effects of this drug. In a rat stroke model, post-insult treatments with therapeutic doses of lithium or valproate markedly reduce ischemia-induced brain infarction and

neurological deficits. These neuroprotective effects are associated with suppression of caspase-3 activation and induction of chaperone proteins. In a rat excitotoxicity model of Huntington's disease in which an excitotoxin was infused into the striatum, both long- and short-term pretreatment with lithium reduces excitotoxin-induced DNA damage, caspase-3 activation, and striatal neuronal loss. This neuroprotection is associated with upregulation of Bcl-2 in neurons. Lithium also induces cell proliferation near the injury site with a concomitant loss of proliferating cells in the subventricular zone. Some of these proliferating cells display neuronal or astroglial phenotypes. This finding corroborates in vitro results showing that lithium stimulates the proliferation of neuroblasts in primary cultures of CNS neurons.

Inositol depletion is an acute response to lithium [74] and valproate [75], and aberrant inositol metabolism has been reported in bipolar patients [76]. The role of inositol as a metabolic sensor for the secretory, unfolded protein response and glucose response pathways suggests that treatment with inositol-depleting drugs may lead to dramatic changes in complex cellular processes [77]. One such recent example is a study by Brandish et al. [78], wherein the authors report highly significant upregulation of the expression of a hypothalamic peptide (PACAP) mediator of monoamine signaling and circadian rhythm in rat brain slices exposed to chronic lithium at clinically relevant concentrations under myo-inositol-limiting conditions. Genetic, molecular, and biochemical studies in yeast for over three decades have elucidated intricate mechanisms underlying regulation of inositol biosynthesis in this model eukaryote. *INO1*, the gene encoding 1D-myo-inositol 3-phosphate (MIP) synthase, is the most highly regulated of the genes required for phospholipid biosynthesis. This enzyme, which catalyzes the rate-limiting step in inositol biosynthesis, is highly conserved from yeast to humans. The human *INO1* gene is functional in yeast and complements the inositol deficiency of the yeast *ino1* mutant. Similar to the yeast enzyme, human MIP synthase activity is decreased in the presence of valproate [79]. Because inositol is a metabolic sensor for a variety of signal transduction pathways, MIP synthase may represent a target for new mood-stabilizing drugs.

22.7 MECHANISM OF ANTICONVULSANT ANTIBIPOLAR COMPOUNDS

Anticonvulsant drugs such as valproate, carbamazepine, and lamotrigine possess antibipolar properties in the clinical setting. Their mechanism in epilepsy most probably involves voltage-activated sodium channels, but effects on GABA, on NMDA receptors, on K^+ channels, and on calcium channels may also be involved in some drugs of this class [80]. Attempts to look for commonalities between lithium and the anticonvulsant antibipolar drugs may be heuristic; however, there may be serious pitfalls as well. For instance, lamotrigine may have efficacy preferentially in the treatment of bipolar depression, lithium appears to have proconvulsant properties in some animal models, and the evidence for long-term efficacy in preventing recurrent episodes of mania and depression may vary among these drugs. As noted earlier and described below, ongoing studies are identifying molecular pharmacological properties that may converge under further investigation. Furthermore, in our future explorations, we should be cautious regarding previously accepted assumptions. For instance, phenytoin in the past was used as a negative control in

biochemical experiments identifying pathways for mood stabilizers, but recent clinical work suggests that this may not be the case [81, 82].

Using a differential display polymerase chain reaction (PCR), Wang and Young found [47] that chronic treatment with valproate at a therapeutically relevant concentration increased expression of 78-kDa glucose-regulated protein (GRP78), an endoplasmic reticulum (ER) stress protein, in rat brain. Chronic valproate treatment increased not only GRP78 mRNA level but also GRP78 gene transcription and protein levels. Chronic treatment with lithium also increased GRP78 mRNA and protein levels in primary cultured rat cerebral cortical cells. Unlike the classic GRP78 inducer thapsigargin, an inhibitor of the ER Ca^{2+} -ATPase, both lithium and valproate moderately increase GRP78 expression in neuronal cells without affecting basal intracellular free Ca^{2+} concentration and cell viability, indicating that these two drugs increase GRP78 expression without causing cell stress. Since GRP78 exhibits molecular chaperone activity, binds calcium, folds damaged proteins, and has been shown to inhibit oxidative stress and apoptosis, their findings corroborate the notion that both lithium and valproate generate a neuroprotective effect against cell damage. Expression of two other closely related ER stress proteins, GRP94 and calreticulin, has also been shown to increase by chronic treatment with lithium and valproate. One may wonder, however, whether upregulation of stress-induced proteins is not merely a nonspecific response to the aversive effect of the drugs.

22.8 CONCLUSION

Despite all the information presented in this chapter, the statement that “the biochemical basis for mood stabilizer therapies or the molecular origins of bipolar disorder is unknown,” which appears in all editions of the Goodman and Gilman textbook and in most of the reviews on mood stabilization and BPD in recent years, is still true. It is highly likely that many of the biochemical findings regarding the mechanism of action of lithium will be found to represent components of pathways that play a role in the pathophysiology of BPD. As noted above, some of these may be shared with the anticonvulsants possessing antibipolar properties. As the allegory of the blind men and the elephant would suggest, at best we are merely looking at parts of the whole. On the other hand, the ultimate understanding of the pathways contributing to the long-term prophylactic action of drugs like lithium in the treatment of BPD is expected to lead to a better understanding of the pathophysiology of BPD and a rational basis for the development of novel antibipolar drugs in the future. In addition, the current advances in neuroimaging for both PET and MRI offer an ideal opportunity to utilize convergent data from our molecular pharmacological studies that will provide promising insights into the underlying neurobiology of this disease.

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INDEX

- Abecarnil, GABA_A subunit pharmacology,
 - allosteric ligand modulation, 503–506
- Acetylcholine (ACh)
 - action potential research on, 26–27
 - early research on, 17
 - Feldberg's research on, 20–21
 - H₃ receptor release, 316
 - Loewi's experiments in, 18–19
 - muscarinic receptors
 - activation mechanisms, 155
 - dimerization, 166–167
 - distribution, 149
 - G-proteing coupling properties, 155–156
 - RGS proteins, 158
 - ion channels, 160–162
 - ligand binding mechanisms, 152–154
 - agonists, 152
 - allosteric ligands, 153–154
 - antagonists, 152–153
 - clinical applications, 154
 - MAPK pathways modulation, 159
 - phenotypic mouse analysis, 167–177
 - agonist-induced tremor and hypothermia, 170–171
 - amylase secretion, exocrine pancreas, 175
 - analgesia, 170
 - autoreceptors, 171
 - cardiovascular system, 175–176
 - cytolytic T cells, 177
 - drug abuse effects, 173
 - epileptic seizures, 169
 - food intake stimulation, 171
 - gastric acid secretion, 175
 - inhibitory hippocampal synapse suppression, 172
 - learning and memory functions, 168–169
 - locomotor activity, 169–170
 - nucleus accumbens, dopamine effects, 172–173
 - pancreatic islet insulin and glucagon secretion, 174–175
 - peripheral autoreceptors and heteroreceptors, 176
 - prepulse inhibition and haloperidol-induced catalepsy, 172
 - salivary secretion, 174
 - skin functions, 177
 - smooth muscle functions, 173–174
 - striatal dopamine release modulation, 171–172
 - regulatory mechanisms, 162–166
 - downregulation, 163–164
 - internatlizations, 162–163
 - phosphorylation, 164–166
 - resensitization, 166
 - uncoupling, 162
 - research background, 148–149

- signaling pathways, 158–159
 - structural features, 149–151
 - neuropeptide electrophysiology and, 676–677
 - neurotransmitter transporters
 - choline, 714–715
 - vesicular transporters, 716–717
 - Torpedo electric organ, 109–110
 - transmitter inactivation, 51–52
 - Acetylcholine binding protein (AChBP),
 - GABA_A receptor activation, agonist binding site architecture, 495
 - Acetylcholinesterase
 - early research on, 23
 - nicotinic acetylcholine receptor and, 28
 - Action potentials
 - early research on, 25–27
 - voltage-gated potassium channels, K_v
 - subunits, structure and function, 628–630
 - Activin, vascular endothelial growth factor and, 809
 - Addiction. *See* Substance abuse
 - Adenosine diphosphate (ADP) ribosylation factor (ARF) family, GABA_B
 - receptor trafficking and heteromization, 577–578
 - Adenosine triphosphate (ATP)
 - plasma membrane glutamate transporters, 709–711
 - second messengers and, 31–32
 - synapse research and, 23
 - Adenylate cyclase
 - GABA_B receptors, effector systems, 582–583
 - H₃ receptor, 314–315
 - lithium mechanism in bipolar disorder patients, 868–869
 - Adenylyl cyclase (AC)
 - α₂ adrenergic receptor regulation, signal transduction pathways, 206
 - cyclic nucleotide second messenger signaling, 72–77
 - heterotrimeric G proteins, 62–63
 - muscarinic acetylcholine receptors,
 - G-protein-coupling properties, 156
 - Adrenaline, early research on, 15–16
 - Adrenergic receptors. *See also* α and β-adrenergic receptors
 - historical perspective, 194–195
 - α-adrenergic receptors
 - α₁-adrenergic receptor
 - general characteristics, 201–203
 - physiological roles, 203–204
 - signal transduction pathways, 203
 - α₂-adrenergic receptor
 - general characteristics, 204–206
 - physiological roles, 207–209
 - signal transduction pathways, 206
 - historical perspective, 196
 - subdivision of, 196
 - β-adrenergic receptors
 - general characteristics and regulation, 209–210
 - physiological roles, 211–213
 - signal transduction pathways, 210–211
 - subdivision of, 195–196
 - Adrenergic transmitters, early research on, 29–30
 - Adrenocorticotrophic hormone (ACTH),
 - neuropeptides and, 670–671
 - behavioral techniques, 677–678
 - opioid family gene duplication, 680–682
- Afferents, histaminergic neurons, 303–305
- Affinity mechanisms, GABA_A receptors,
 - desensitization and deactivation, 498–499
- Afterhyperpolarization (AHP),
 - small-conductance calcium-activated potassium channels, 631–632
- Agatoxins, voltage-gated calcium ion channels, Ca_v2 family, 649
- Agonist chelation hypothesis, GABA_A
 - receptor activation, 493–494
 - binding site architecture, 494–495
- Agonists
 - α₁ adrenergic receptors, 202–203
 - GABA_A receptor activation, 492–495
 - allosteric ligand modulation, 500–506
 - glutamate transporter regulation, 728
 - muscarinic acetylcholine receptor, ligand binding, 152
 - neuropeptide receptors, 675
 - serotonin receptor families, 263–267
 - upregulation, nicotinic acetylcholine receptor, 127–130
- A kinase anchoring proteins (AKAPs),
 - GABA_A phosphorylation, 482
- Alcohols, GABA_A receptor modulation, 511–516

- Aldo-keto reductase family, voltage-gated potassium channels, 627–628
- Allopregnanolone, GABA_A receptor modulation, 515
- Allosteric ligands
 - GABA_A receptor modulation
 - benzodiazepine recognition site, 499–506
 - benzodiazepine structural determinants, 507–509
 - transmembrane domains, 516–518
 - unidentified sites, 519–520
 - GABA_B receptor subunits, 580
 - muscarinic acetylcholine receptor, 153–154
- Allosteric potentiators and antagonists
 - GABA_B receptors, 590–592
 - ionotropic glutamate receptors, 385–397
 - AMPA antagonists, 395–396
 - AMPA potentiators, 390–395
 - kainate receptor antagonists, 397
 - kainate receptor potentiators, 396
 - NMDA receptor antagonists, 386–390
 - NMDA receptor potentiators, 385–386
 - metabotropic glutamate receptors
 - mGlu1 antagonists, 436–437
 - mGlu2/3 antagonists, 439–440
 - mGlu5 antagonists, 443–445
 - mGlu7 antagonists, 445
 - mGlu1 potentiators, 435–436
 - mGlu2 potentiators, 437–439
 - mGlu4 potentiators, 440–441
 - mGlu5 potentiators, 441–443
- α_1 interaction domain (AID), voltage-gated calcium channels, 644
- Alzheimer's disease
 - dopaminergic drugs, 237
 - GABA_A α_5 subunit pharmacology, 489
 - histaminergic neuron activity, 327–328
 - KCNQ channels and, 633
 - nitric oxide and, 754
 - plasma membrane glutamate transporter, 719
- Amino acid residues, GABA_A receptors
 - activation, agonist binding site architecture, 494–495
 - assembly, 478
 - modulation, allosteric structural determinants, 508–509
- Amino acid transmitters
 - early research on, 32
 - intercellular signaling and, 42–44
- 1-Amino-cyclopentane-1,3-dicarboxylic acid (ACPD), mGlu group I selective orthosteric agonists, 423
- Aminocyclopropylcarboxylic acid (ACC), NMDA allosteric potentiators, 386
- (*R,S*)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), mGlu I selective orthosteric antagonists, 425
- 2*R,4R*-4-Aminopyrrolidine-2,4-dicarboxylate (2*R,4R*-APDC), mGlu II selective orthosteric agonists, 429
- AMPA receptors
 - allosteric antagonists, 395–396
 - allosteric potentiators, 390–395
 - kainate receptor orthosteric agonists, 381
 - mGlu group I selective orthosteric agonists, quisqualate, 423
 - orthosteric agonists, 376–377
 - orthosteric antagonists, 377–378
 - structure and function, 370–371
- Amphetamine derivatives, serotonin synthesis, 261
- Amylase secretion, exocrine pancreas, muscarinic acetylcholine receptor deficiency, 175
- Amyotrophic lateral sclerosis (ALS)
 - nitric oxide and, 754
 - plasma membrane glutamate transporter, 718–719
- Analgesia
 - α_2 adrenergic receptor physiology, 207–208
 - muscarinic acetylcholine receptor deficiency, 170
- Anesthesias
 - GABA_A β subunit pharmacology, 490–491
 - GABA_A receptor modulation, 511–516
 - voltage-gated sodium ion channels and, 643
- Angina therapy, voltage-gated calcium channels, Ca_v1 family, 648
- Angiotensin-converting enzyme (ACE), neuropeptide inactivation, 687–688
- Animal models
 - antidepressants and hippocampal neurogenesis, 833
 - curare research on, 5–7
 - pharmacology research and, 11
- Aniracetam, AMPA receptor allosteric potentiators, 390–391

Antagonists

- GABA_A receptor activation, 492–495
 - allosteric ligand modulation, 500–506
- GABA_B receptors, 590
- glutamate transporter regulation, 728
- muscarinic acetylcholine receptor, ligand
 - binding, 152–153
- neuropeptide receptors, 675

Anxiolytic drugs

- GABA_A subunit pharmacology
 - allosteric ligand modulation, 503–506
 - α subunits, 483–490, 509–511
 - α_1 subunit, 484, 488
 - α_2 subunit, 488
 - α_3 subunit, 488
 - α_4 subunit, 488–489
 - α_5 subunit, 489
 - α_6 subunit, 489–490
 - electrophysiology, 486–487
 - modulator selectivity, 484
- terminology, 483
- GABA_B receptor targeting, 594–595
- 5-HT_{2C} receptors and, 272–273
- mGlu5 allosteric antagonists, 443–444
- mGlu2 allosteric potentiators, 439
- mGlu II selective orthosteric agonists, 427–429
- mGlu II selective orthosteric antagonists, 430–431
- serotonin receptors, 263–266

Antiarrhythmics

- KCNH potassium channels, 634
- voltage-gated calcium channels, Ca_v1
 - family, 648
- voltage-gated sodium ion channels and, 642

Anticonvulsant drugs

- AMPA receptor allosteric antagonists, 396
- in bipolar disorder patients, 869–870
- GABA_A α_1 subunit pharmacology, 484–485, 488
- GABA_A receptor modulation, 511–516
- GABA_B receptor targeting, 595
- histamine neuron physiology, 326
- mGlu II selective orthosteric agonists, 428–429
- mGlu I selective orthosteric antagonists, 425–426

Antidepressants

- β -adrenergic receptor activation, 210
- GABA_B receptor targeting, 594–595

hippocampal neurogenesis and

- adult neurogenesis stages, 827–828
- behavioral and animal models, 833
- depression physiology and, 823–824, 826–827
- future research on, 833
- monoamines and, 822–823
- research background, 821–822
- serotonin/norepinephrine regulation, 831–833
- stress and monoamine hypotheses, 825–826
- stress hormone neurogenesis regulation, 829–830

histaminergic neuron activity, 329

5-HT_{2C} receptors and, 272–273

mGlu II selective orthosteric antagonists, 431

neurotrophic factor expression and, 804–806

BDNF expression, 804–805

models for, 805–806

neurogenesis in adults and, 806–808

serotonin receptors, 263–266

Antidiabetic agents, β -adrenergic receptor physiology, 212–213Antidiuretic hormone (ADH). *See* Vasopressin

Antihistamines

- arousal mechanisms, 323–324
- KCNH potassium channels, 634

Antiobesity agents, β -adrenergic receptor physiology, 212–213

Antipsychotic drugs

- α_2 -adrenergic receptor physiology, 209
- in bipolar disorder patients, 869–870
- histaminergic neuron activity, 327
- 5-HT₆ receptor family and, 275
- mGlu2 allosteric potentiators, 439
- mGlu II selective orthosteric agonists, 427–429

Anxiety

- histaminergic neuron activity, 329
- 5-HT_{2B} receptors and, 271
- serotonin transporters and, 720

Apamin, small-conductance

- calcium-activated potassium channels, 632

AP-1 complex, amino acid

- neurotransmitters, 44

Appetite regulation

- histaminergic neuron activity, 325–326

- 5-HT_{2C} receptors and, 272–273
- muscarinic acetylcholine receptor deficiency, 171
- Arginine vasopressin, neuropeptide receptors, 689
- Aromatic amino acid decarboxylase (AAAD)
 - dopamine synthesis, 223–224
 - serotonin synthesis, 260–261
- Arousal, histamine neuron physiology, 322–324
- Arrhythmias
 - KCNH potassium channels, 634
 - voltage-gated calcium channels, Ca_v1 family, 648
 - voltage-gated sodium ion channels and, 642
- 2-Arylureidobenzoic acids, kainate receptor allosteric antagonists, 397
- Assembly mechanisms, GABA_A receptor assembly, 477–478
- ATPA, kainate receptor orthosteric agonists, 381
- Atropine, early research on, 10
- Attention-deficit hyperactivity disorder (ADHD)
 - dopaminergic drugs, 237–238
 - dopamine transporters and, 721
 - GABA_B receptor targeting, 592–593
 - histaminergic neuron activity, 329
- Auditory function, KCNQ channel action on, 632–633
- Autism
 - dopaminergic drugs, 237
 - serotonin transporters and, 720
- Autoimmune diseases, nitric oxide physiology, 745
- Autonomic nervous system, early research on, 13
- Autoradiography, neuropeptide identification, 673–674
- Autoreceptors
 - chemical transmission, 50–51
 - H₃ receptor, 314–315
 - muscarinic acetylcholine receptor deficiency, 171
 - peripheral agents, 176
 - serotonin receptor families, 263–267
- Backpropagation action potentials, voltage-gated potassium channels,
 - K_v subunits, structure and function, 629–630
- Baclofen
 - GABA_B receptors, 469
 - anxiety and depression therapy, 594–595
 - targeting, 592–593
- Barium
 - second messengers and, 31–32
 - voltage-gated potassium channels, K_v subunits, structure and function, 630
- BAY367620, mGlu1 allosteric antagonists, 437
- Behavioral techniques
 - antidepressants and hippocampal neurogenesis, 833
 - neuropeptide electrophysiology and, 677–678
 - neurotrophic factors and, 808–809
- Benign neonatal familial convulsion (BNFC), KCNQ channels and, 633
- Benzamides
 - AMPA receptor allosteric potentiators, 390
 - NMDA receptor allosteric antagonists, 390
- Benzimidazoles, NMDA receptor allosteric antagonists, 390
- Benzodiazepine
 - GABA_A receptor modulation
 - allosteric binding, 499–506
 - allosteric structural determinants, 507–509
 - binding sites, 506–507
 - GABA_A subunit pharmacology
 - α subunits, 483–490, 509–511
 - α_1 subunit, 484, 488
 - α_2 subunit, 488
 - α_3 subunit, 488
 - α_4 subunit, 488–489
 - α_5 subunit, 489
 - α_6 subunit, 489–490
 - electrophysiology, 486–487
 - modulator selectivity, 484
 - γ subunit isoforms, 509–511
 - terminology, 483
- 2,3-Benzodiazepine, AMPA receptor allosteric antagonists, 395
- Benzothiadiazides, AMPA receptor allosteric potentiators, 390–392

- Biarypropylsulfonamides, AMPA receptor allosteric potentiators, 393
- Bicuculline, GABA_A receptor inhibition, 468–469
- Binding affinities, GABA_A subunit selectivity, 484–485
- Bioassays, neuropeptide identification, 671
- Biosynthesis
 - classical neurotransmitters, 42–43
 - histamine metabolism, 305–306
 - neuropeptides, 685–687
 - preprohormones, 686
 - prohormones, 686
 - tissue specificity, 687
- Biotini-switch method, nitrosothiol detection, 748
- Bipolar disorder
 - dopaminergic drugs, 237
 - neurobiology of
 - genetics, 860–861
 - peripheral neurochemistry, 863–864
 - postmortem findings, 861–863
 - research background, 859
 - in vivo imaging, 863
 - pharmacology
 - anticonvulsant antibipolar compounds, 869–870
 - lithium mechanism of action, 864–869
- Blood-brain barrier, neuropeptides in, 692–693
- Blood pressure, norepinephrine/epinephrine physiology and, 197–198
- Blood vessels, muscarinic acetylcholine receptor deficiency, 176
- Bovine rhodopsin, muscarinic acetylcholine receptor structure, 149–151
- Bphs* gene, H₁ receptor in, 307–309
- Brain-derived neurotrophic factor (BDNF)
 - AMPA receptor allosteric potentiators, 394–395
 - antidepressant treatments and expression of, 804–805
 - adult neurogenesis and, 806–808
 - 5-HT neuronal growth and, 807–808
 - stress-monoamine convergence in depression, 825–826
 - in depressed patients, 800–801
 - depression models and, 808
 - GABA_A phosphorylation, 482
 - genetic studies in mood disorders, 803
 - intracellular signaling, 791–792
 - lithium mechanism in bipolar disorder and, 868–869
 - stress influence on, 794
 - adrenal glucocorticoids, 796–797
 - alterations in, 794–795
 - cytokine alterations, 797
 - neurogenesis and, 799–800
 - regulation mechanisms, 795–796
 - serotonin receptors, 797
- Brain development
 - in bipolar disorder patients, 860–861
 - GABA_A receptor subunit distribution and, 471–473
 - GABA transporters and, 467–468
 - hippocampal neurogenesis and depression and, 826–827
 - voltage-gated sodium ion channels, 640–641
- Brain imaging studies, depressed patients, 802
- Brain neurotransmitters, histamine
 - biosynthesis, 305–306
 - early history, 300–301
 - inactivation, 306–307
 - metabolism, 305–307
 - molecular pharmacology, receptor subtypes, 307–317
 - H₁ receptor, 307–312
 - H₂ receptor, 312–314
 - H₃ receptor, 314–316
 - H₄ receptor, 316
 - NMDA receptor interaction, 316–317
 - neuron activity and control, 317–322
 - electrophysiology, 317
 - pharmacological changes, 321–322
 - in vitro modulation, 317–319
 - in vivo changes, 319–321
 - neuronal organization, 301–305
 - afferents, 303–305
 - histaminergic pathways, 303
 - perikarya, 301–303
 - neuron physiology, 322–326
 - arousal, 322–324
 - cognitive functions, 324
 - nociception, 326
 - pituitary hormone secretion, 324–325
 - satiating, 325–326
 - seizures, 326
 - neuropsychiatric disease, 327–329
 - Alzheimer's disease, 327–328
 - anxiety/ADHD, 329

- Parkinson's disease, 328
- schizophrenia and antipsychotic actions, 327
- Bretazenil, GABA_A subunit pharmacology,
 - allosteric ligand modulation, 504–506
- Brugada syndrome, voltage-gated sodium ion channels and, 642
- α -Bungarotoxin, nicotinic acetylcholine receptor and, 28
- γ -Butyrolactones, GABA_A receptor modulation, 519–520
- Calcium-activated potassium channels,
 - structure and function, 630–632
- Calcium-calmodulin-dependent protein kinase II
 - amino acid neurotransmitters, 43–44
 - GABA transporters, 467–468
- Calcium channels
 - amino acid neurotransmitters, 43–44
 - in bipolar disorder patients, 862
 - calmodulin mediator, 85–87
 - cyclic nucleotide second messenger signaling, adenylyl cyclase, 72–77
 - GABA_B receptors, effector systems, 581–582
 - histaminergic neuron activity, 317–319
 - H₁ receptor signaling, 309
 - muscarinic acetylcholine receptor modulation, 161
 - signaling molecules, 83–85
 - voltage-gated ion channels, 643–650
 - α subunits, 644–645
 - $\alpha\delta$ subunits, 646
 - auxiliary subunit modulators, 650
 - β subunits, 645–646
 - Ca_v1 family, 647–648
 - Ca_v2 family, 648–649
 - Ca_v3 family, 649–650
 - γ subunits, 646
 - general blockers, 647
 - genetics, 619–620
 - miscellaneous channels, 650–651
- Calcium metabolism, 30–31
- Calmodulin
 - calcium channel modulation, 85–87
 - Ca_v1 family, 647–648
 - intermediate-conductance
 - calcium-activated potassium channels, 631
- CamKII, GABA_A receptor
 - phosphorylation, 481–482
- cAMP response element binding (CREB) protein
 - amino acid neurotransmitters, 44
 - BDNF regulation and, 805
 - calcium channel modulation, Ca_v1 family, 647–648
 - lithium mechanism in bipolar disorder and, 868–869
- CA1 pyramidal cells, GABA_A receptor distribution on, 474–475
- CA3 pyramidal neurons, stress and, 800
- Carbon monoxide, as signaling molecule, 756–757
- 2-(Carboxycyclopropyl)glycines (CCGs), mGlu II selective orthosteric agonists, 426
- S4-Carboxy phenylglycine (*S*-4CPG), mGlu group I selective orthosteric antagonists, 424–425
- Cardiovascular system. *See also* Arrhythmias
 - β -adrenergic receptor physiology, 211–212
 - α_2 -adrenergic receptor physiology, 208–209
 - KCNQ channel action on, 632–633
 - muscarinic acetylcholine receptor deficiency, 175–176
 - voltage-gated calcium channels, 647–648
 - voltage-gated sodium ion channels, 640–641
- Carrier-mediated mechanisms, blood-brain barrier neuropeptides and, 692–693
- Catalepsy, muscarinic acetylcholine receptor deficiency, 172
- Catecholamines, early research on, 30–31
- Catechol-*O*-methyltransferase (COMT)
 - dopamine synthesis, 224
 - norepinephrine neurochemistry, 198–199
- Cation channels, muscarinic acetylcholine receptor modulation, 161
- CDPPB, mGlu5 allosteric potentiators, 442–443
- Cell survival, nitrosylation and, 752
- Cellular pathways, nitrosylation in
 - cell survival, 752
 - extracellular matrix, 751–752
 - gene transcription, 750–751
 - ion channels, 748–749
 - protein-protein interactions, 749–750
 - vesicular transport, 751

- Central nervous system (CNS)
 β -adrenergic receptor physiology, 211–212
 adrenergic transmitters in, 29–30
 AMPA receptors, 370–371
 GABA_A receptor subunit distribution in, 471–473
 5-HT₇ receptor family in, 275–276
 5-HT₄ receptor family in, 274
 5-HT₃ receptors and, 273
 kainate receptors, 372
 NMDA receptors, 369–370
 serotonin impact on, 258–259
 voltage-gated calcium ion channels, Ca_v2 family, 648–649
 voltage-gated sodium ion channels, 640–641
- Cerebrospinal fluid (CSF), blood-brain barrier neuropeptides and, 692–693
- Cesium, voltage-gated potassium channels, K_V subunits, structure and function, 630
- Channel blockers
 GABA receptor binding sites, 518–519
 NMDA receptor allosteric antagonists, 387–389
 voltage-gated calcium ion channels, Ca_v2 family, 649
- Channel-gating process, GABA_A receptor activation, 492–494
 binding transduction to, 495–497
- Channelopathies, voltage-gated sodium ion channels, 641–642
- Channel pore structures, GABA_A receptor activation, 497–498
- Charybdotoxin, intermediate-conductance calcium-activated potassium channels, 631
- Chemical neurotransmitters, categories of, 40–41
- Chimeric receptors, GABA_A receptor modulation, allosteric structural determinants, 508–509
- Chlordiazepoxide (CDPX), GABA_A receptor modulation, 499–500
- Chloride channels
 early research on, 29
 GABA receptors and, 468–469
 muscarinic acetylcholine receptor modulation, 161–162
 voltage-gated channels, 649–650
- (R,S)-2-Chloro-5-hydroxyphenylglycine (CHPG), mGlu group I selective orthosteric agonists, 424
- Cholecystokinin (CKK), neuropeptides and, 670–671
- Choline acetyltransferase (CHAT), structure and function, 714–715
- Choline neurotransmitter transporters (CHT), structure and function, 714–715
- Chrysin, GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
- Ciproxifan, histamine neuron physiology and, 324
- Circadian rhythms
 histaminergic neuron activity, 319–322
 reproductive cycle depression
 future research issues, 852–853
 neuroendocrine compounds
 cross-reproductive cycle analysis, 851–853
 menopause, 849–851
 cortisol, 850
 melatonin, 849–850
 prolactin, 850–851
 thyroid-stimulating hormone, 850
 menstrual cycle, 844–846
 cortisol, 845
 melatonin, 844–845
 prolactin, 846
 thyroid-stimulating hormone, 845
 postpartum depression, 848–849
 cortisol, 848
 estradiol, 849
 melatonin, 848
 prolactin, 848–849
 thyroid-stimulating hormone, 848
 pregnancy, 846–847
 cortisol, 847
 melatonin, 846–847
 prolactin, 847
 thyroid-stimulating hormone, 847
 research background, 844
- CL-218, 872, GABA_A receptor activation, benzodiazepine binding sites, 510–511
- Clathrin-mediated endocytosis, GABA_A receptor trafficking, 480–481
- Clonazepam, GABA_A α_3 subunit pharmacology, 488

- Cloning experiments
 - cyclic nucleotide second messenger signaling, adenylyl cyclase, 74–75
 - GABA_B receptors, 571–572
 - glutamate receptors, 397–398
 - NMDA receptors, 369–370
 - voltage-gated ion channels, 619–620
- Clostridium botulinum*, synaptic vesicle action and, 27
- Clotrimazole, intermediate-conductance calcium-activated potassium channels, 631
- Clozapine, vesicular transporters and, 723
- Clustering behavior, GABA_A receptors, 478–480
- Cocaine- and amphetamine-regulated transcript (CART), stress response and, 695
- Cognitive functions
 - GABA_A α_5 subunit pharmacology, 489
 - GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
 - GABA_B receptor targeting, 592–593
 - histamine neuron physiology, 324
- Colorimetry, nitrosothiol detection, 748
- Complexins, in bipolar disorder patients, 861–862
- Concanavlin A, kainate receptor allosteric potentiators, 396
- Congenital stationary night blindness (CSNB2), voltage-gated calcium channels, Ca_v1 family, 648
- Conotoxins
 - nicotinic acetylcholine receptors, 124
 - voltage-gated calcium ion channels, Ca_v2 family, 649
- COPI components, GABA_B receptor trafficking and heteromization, 576–578
- Corticotropin-releasing hormone (CRH), stress response and, 694–695
- Cortisol, depression and levels of
 - in menopause, 850
 - during menstrual cycle, 845
 - postpartum depression, 848
 - in pregnancy, 846–847
- CPCCOEt, mGlu1 allosteric antagonists, 436–437
- CPPHA, mGlu5 allosteric potentiators, 442
- Curare
 - action of, 4–5
 - Bernard's research on, 4–7
- CX546, AMPA receptor allosteric potentiators, 390–391
- CX614, AMPA receptor allosteric potentiators, 393–395
- Cycle regulation, G proteins, 64–65
- Cyclic adenosine monophosphate (cAMP)
 - α_1 adrenergic receptors, 203
 - in bipolar disorder patients, 861–862
 - cyclic nucleotide second messenger signaling, 75, 78
 - second messengers and, 32
- Cyclic guanosine monophosphate (cGMP)
 - cyclic nucleotide second messenger signaling, cellular targets, 80–81
 - GABA_A phosphorylation, 481–482
 - nitric oxide physiology, 745
- Cyclic nucleotide-gated (CNG) channels, structure and function, 649–650
- Cyclic nucleotide second messengers, intracellular signaling, 72–81
 - adenylyl cyclase, 72–77
 - cAMP targets, 75, 78
 - cGMP targets, 80–81
 - guanylyl cyclase, 78–80
- Cyclopropylglutamate (CCG), NMDA orthosteric agonists, 373
- (*R,S*)- α -Cyclopropyl-4-phosphonophenylglycine (CPPG), mGlu III selective orthosteric agonists, 435
- Cyclothiazide, AMPA receptor allosteric potentiators, 390–395
- cyPPTS, mGlu2 allosteric potentiators, 438–439
- Cys loop ligand-gated ion channel superfamily
 - GABA_A receptor activation, 493–494
 - channel pore complex, 497–498
 - GABA receptors and, 468–469
- Cysteine residues, nitric oxide nitrosylation, 747
- Cystinyl leukotriene, mGlu2 allosteric potentiators, 439
- Cytisine, nicotinic acetylcholine receptors, 120
- Cytochemical assay, neuropeptide identification, 673
- Cytokines
 - neuropeptide receptors, 691
 - stress response and, neurotrophins and, 797

- Cytolytic T cells, muscarinic acetylcholine receptor deficiency, 177
- Cytoplasmic proteins
sodium voltage-gated ion channels, 636
voltage-gated potassium channels, 627–628
- Cytoskeletal proteins, voltage-gated potassium channels, 628
- DAG activation, protein kinase C, 85
- D-2-amino-5-phosphonopentonoate (D-AP5), NMDA orthosteric antagonists, 373–375
- DARPP-32, protein phosphatases integration, 95–96
- Deactivation, GABA_A receptors, 498–499
- Deafness, KCNQ channels and, 632–633
- Decahydroisoquinolines
AMPA receptor orthosteric antagonists, 377–378
kainate receptor orthosteric antagonists, 383–385
- Decarboxylation, dopamine synthesis, 54–55
- Degradation reactions, serotonin, 261–262
- Dendrotoxins, voltage-gated potassium channels, K_V subunits, structure and function, 630
- Depolarization responses
GABA receptors, 468–469
voltage-gated calcium channels, 647–648
voltage-gated sodium ion channels, 640
- Depression
hippocampal neurogenesis and treatment for
adult neurogenesis stages, 827–828
behavioral and animal models, 833
depression physiology and, 823–824, 826–827
future research on, 833
monoamines and, 822–823
research background, 821–822
serotonin/norepinephrine regulation, 831–833
stress and monamine hypotheses, 825–826
stress hormone neurogenesis regulation, 829–830
neurotrophic factors in, 800–802
antidepressant therapies
BDNR expression and, 804–806
neurogenesis and, 806–808
BDNF levels, 800–801
- Brain-derived neurotrophic factor and models of, 808
fibroblast growth factor levels, 802
structural and cellular alterations, 802–803
in reproductive cycle, circadian studies of neuroendocrine compounds
cross-reproductive cycle analysis, 851–853
future research issues, 852–853
menopause, 849–851
cortisol, 850
melatonin, 849–850
prolactin, 850–851
thyroid-stimulating hormone, 850
menstrual cycle, 844–846
cortisol, 845
melatonin, 844–845
prolactin, 846
thyroid-stimulating hormone, 845
postpartum depression, 848–849
cortisol, 848
estradiol, 849
melatonin, 848
prolactin, 848–849
thyroid-stimulating hormone, 848
pregnancy, 846–847
cortisol, 847
melatonin, 846–847
prolactin, 847
thyroid-stimulating hormone, 847
research background, 844
serotonin transporters and, 720
- Desensitization
 α_1 adrenergic receptors, 202–203
 β -adrenergic receptor activation, 210
AMPA receptor allosteric potentiators, 393–395
GABA_A receptors, 498–499
GABA_B receptors, 584–585
neuropeptide receptors, 688–689
of nicotinic acetylcholine receptor, 125–127
- Diabetes mellitus, β -adrenergic receptor physiology, 212–213
- Diazepam
GABA_A α_1 subunit pharmacology, 484–485, 488
GABA_A receptor modulation, 499
 γ subunit isoforms, 509–511

- 2',3'-Dicarboxycyclopropylglycine analog (DCG-IV), mGlu II selective orthosteric agonists, 426–427
- 3,3'-Difluorobenzaldazine, mGlu5 allosteric potentiators, 441–442
- Dihydroxyphenylalanine (DOPA), norepinephrine/epinephrine neurochemistry, 198–199
- 3,5-Dihydroxyphenylglycine (3,5-DHPG), mGlu group I selective orthosteric agonists, 423–424
- Diisopropyl phosphorofluoridate (DFP), synapse research and, 23
- Dimerization
 α_1 adrenergic receptors, 201–203
 muscarinic acetylcholine receptors, 166–167
- Dipeptidyl-peptidase IV (DPP IV), neuropeptide inactivation, 687–688
- Disease, G proteins and, 66
- DMPP agonist, nicotinic acetylcholine receptors, 120
- DOPA (3,4-dihydroxyphenylalanine), early research on, 30–31
- Dopamine
 histaminergic neuron activity, Parkinson's disease, 328
 H_3 receptor synthesis, 315–316
 muscarinic acetylcholine receptor deficiency
 efflux nucleus accumbens, 172–173
 striatal release, 171–172
 neurotransmission
 attention-deficit hyperactivity disorder, 237–238
 basic properties, 221–222
 bipolar disorders, 237
 chemistry and metabolism, 222–224
 classification and molecular properties, 225–230
 D_1 receptor subfamily, 225–227
 D_2 receptor subfamily, 227–230
 drug applications, 236–239
 mechanisms, 54–55
 Parkinson's disease, 236
 polymorphisms, splice variants and SNPs, 234–236
 schizophrenia, 237
 signal transduction pathways, 230–232
 structure-affinity/structure-activity relationships, 232–234
 substance abuse, 238
 transporters
 chronic substrate treatment, 726
 clinical relevance, 721
 multiple interactions, 723–724
 polymorphisms, 724–725
 structure and function, 712–713
- Downregulation
 muscarinic acetylcholine receptors, 163–164
 neuropeptide receptors, 688–689
- Downstream signaling molecules, ion channel modulation, 69–70
- D_1 receptor, subfamily, 225–227
- D_2 receptor, subfamily, 227–230
- D_3 receptor subunit, properties of, 229–230
- D_4 receptor subunit, properties of, 230
- Drug abuse. *See* Substance abuse
- Drug interactions
 α_1 adrenergic receptors, 202–203
 dopaminergic drugs, 236–239
 attention-deficit hyperactivity disorder, 237–238
 bipolar disorders, 237
 Parkinson's disease, 236
 schizophrenia, 237
 muscarinic acetylcholine receptors, 154
- Dysiherbaine, kainate receptor orthosteric agonists, 380
- EAG genes, KCNH potassium channels, 633–634
- Effector systems, GABA_B receptors, 580–583
 adenylate cyclase, 582–583
 calcium channels, 581–582
 G-protein-dependent/independent GABA_B effects, 580–581
- MAPKs, 583
- potassium channels, 582
- Electrophysiology
 histaminergic neuron activity, 317
 neuropeptides, 675–677
 neurohormones, 675–676
 neuromodulators, 676–677
 neurotransmitters, 676
 potassium channels, 625
- Electrospray ionization mass spectrometry (ESI-MS), nitrosothiol detection, 747–748
- ELK genes, KCNH potassium channels, 633–634
- Endocannabinoids

- packaging of, 46
- synthesis of, 45
- Endocytosis, neuropeptide receptor
 - downregulation, 688–689
- Endothelium-derived relaxing factor (EDRF), nitric oxide and, 743–745
- End-plate potentials, early research on, 26–27
- Enzymes, neurotransmitter biosynthesis, 42–43
- Epibatidine, nicotinic acetylcholine receptors, 121
- Epileptic seizures
 - GABA_A β subunit pharmacology, 490–491
 - GABA_B receptor therapy, 595
 - histamine neuron physiology, 326
 - muscarinic acetylcholine receptor deficiency, 169
 - NMDA orthosteric antagonists, 375–377
 - voltage-gated sodium ion channels and, 642
- Epinephrine
 - α -adrenergic receptors, α_2 -adrenergic receptor, 205–206
 - β -adrenergic receptor activation, 209–210
 - basic properties, 193
 - dopamine synthesis, 223–224
 - historical perspective, 194–196
 - neurochemistry, 198–199
 - physiology, 197–198
- Episodic ataxia type 1 (EA-1), voltage-gated
 - potassium channels, K_V subunits, structure and function, 629–630
- Ergot, early research on, 15–16
- Estradiol, postpartum depression and levels of, 849
- Estrogen receptors, histaminergic neuron activity, 325
- Ethanol
 - GABA_A receptor modulation, 516
 - histaminergic neuron activity, 321–322
- Evolutionary relationships
 - neuropeptides, 679–685
 - gene duplication, 680–685
 - gene splicing, 685
 - neurohypophyseal family, 682–684
 - NPY family, 684
 - opioid family, 680–682
 - structural conservation, 679–680
 - voltage-gated ion channels, 619–620
- Excitatory amino acid transporter (EAAT)
 - glutamate neurotransmitters, 53–54
 - plasma membrane glutamate transporter, clinical relevance, 718–719
 - plasma membrane glutamate transporters, 708–711
- Excitatory postsynaptic potentials (EPSPs)
 - early research on, 28–29
 - mGlu2 allosteric potentiators, 438–439
 - neuropeptides and, 676–677
- Excitatory reponses
 - H₂ receptor, 312–313
 - H₁ receptor signaling, 309–311
- Exocrine pancreas, amylase secretion, muscarinic acetylcholine receptor deficiency, 175
- Exocytosis, neuropeptide electrophysiology, 676
- Extracellular calcium, GABA_B receptor modulation, 584
- Extracellular catabolism, dopamine synthesis, 55
- Extracellular domain (ECD), GABA_B receptors
 - modulation, 585–587
 - sites, 578–579
 - subtypes, 573–575
- Extracellular inactivation, neuropeptides, 687–688
- Extracellular matrix (ECM), nitrosylation and, 751–752
- Extracellular signal-related kinase (ERK)
 - brain-derived neurotrophic factor in depressed patients and, 801
 - GABA_B receptors, effector systems, 583
 - lithium mechanism in bipolar disorder patients, 867–869
 - muscarinic acetylcholine receptor modulation, 159
 - phosphorylation, 89–90
- "Fail-safe" mechanism, stress response and, 695
- Famotidine, histaminergic neuron activity, 327
- Fast inactivation, potassium channels, 627
- Fatty acids, GABA_A receptor modulation, 520
- Fibroblast growth factor (FGF)
 - antidepressant influences on, 805–806
 - in depressed patients, 802
 - intracellular signaling, 793

- Filamin, voltage-gated potassium channels, 628
- FKBP5 cochaperone, stress-monoamine convergence in depression, 825–826
- Flavonoids, GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
- Flip/flop exons
 - AMPA receptor allosteric potentiators, 393
 - AMPA receptors, 370–371
- Flumazenil, GABA_A α_6 subunit pharmacology, 489–490
- Fluorometry, nitrosothiol detection, 748
- Follicle-stimulating hormone, in menopause, 851
- Food intake stimulation
 - histaminergic neuron activity, 320–322
 - muscarinic acetylcholine receptor deficiency, 171
- GABAergic neurons
 - histaminergic neuron activity, 319–322
 - H₂ receptor, 313
- Gabapentin, voltage-gated calcium ion channels, auxiliary subunit modulators, 650
- GABARAP polypeptide, GABA_A receptor trafficking, 478–480
- GABA transporters (GATs), structure and function, 711–712
- GAD protein, in bipolar disorder patients, 862
- Galanin, histaminergic neuron activity, 318–319
- γ -amino butyric acid (GABA) receptors
 - A, B, and C receptor classifications, 468–469
 - adult neurogenesis and, 829
 - agonist binding site architecture, 494–495
 - binding-to-gating transduction, 495–497
 - in bipolar disorder patients, 861–862
 - channel blocker binding sites, 518–519
 - channel pore ion selectivity, 497–498
 - early research on, 32, 466
 - GABA_A receptors
 - allosteric modulation
 - benzodiazepine recognition site, 499–506
 - structural determinants, 507–509
 - α subunit pharmacology, 483–490, 509–511
 - α_1 subunit, 484, 488
 - α_2 subunit, 488
 - α_3 subunit, 488
 - α_4 subunit, 488–489
 - α_5 subunit, 489
 - α_6 subunit, 489–490
 - electrophysiology, 486–487
 - modulator selectivity, 484
 - terminology, 483
 - assembly, 477–478
 - benzodiazepine binding site, 506–507
 - subunit isoforms, 509–511
 - β subunit pharmacology, 490–491
 - δ subunit pharmacology, 491–492
 - desensitization and deactivation, 498–499
 - ϵ subunit distribution, 472, 492
 - γ subunit pharmacology, 491–492, 509–511
 - hetero-oligomeric structure, 476–477
 - homo-oligomeric structure, 476
 - modulation mechanisms, 511–516
 - pharmacology, 482–483
 - phosphorylation, 481–482
 - π subunit distribution, 472, 492
 - rare subunits, 492
 - ρ subunit distribution, 472
 - structure, 475
 - subcellular distribution, 473–475
 - θ subunit distribution, 472, 492
 - trafficking, 478–481
 - transmembrane domain allosteric sites, subunits, 516–518
 - unidentified allosteric modulation, 519–520
- GABA_B receptors
 - deficient mice, 587–588
 - effector systems, 580–583
 - endogenous GABA_B ligands, 588–589
 - expression cloning, 571–572
 - G-protein coupling determinants, 579–580
 - ligand binding sites, 578–579
 - modulation mechanisms, 584–586
 - molecular subtypes, 572–575
 - novel compounds, 590–592
 - structure and function, 570–571
 - trafficking and heteromization, 575–578
- histaminergic perikarya, 302–303
- ion channel modulation, protein-protein interaction, 70–71
- metabotropic receptors

- agonists and competitive antagonists, 589–590
- disease and, 592–597
 - anxiety and depression, 594–595
 - drug addiction, 593–594
 - epilepsy, 595
 - gene linkage studies, 596–597
 - nociception, 595–596
 - therapeutic targeting, GABA_B receptors, 592–593
 - tumor cell growth and migration, 596
- effector systems, 580–583
 - adenylate cyclase, 582–583
 - calcium channels, 581–582
 - G-protein-dependent/independent GABA_B effects, 580–581
 - MAPKs, 583
 - potassium channels, 582
- endogenous GABA_B ligands, 588–589
- GABA_B deficient mice, 587–588
- GABA_B novel compounds, 590–592
- GABA_B receptor modulation, 584–586
 - extracellular calcium, 584
 - interacting proteins, 585–587
 - phosphorylation and desensitization, 584–585
- research background, 570–571
- structural properties, 571–580
 - allosteric interactions, 580
 - expression cloning, 571–572
 - G-protein coupling determinants, 579–580
 - liganding binding sites, 578–579
 - molecular subtypes, 572–575
 - surface trafficking and heteromerization, 575–578
- neuropeptides and, 671
- plasma membrane neurotransmitter transporters
 - clinical relevance, 721–722
 - interacting protein regulation, 728
 - structure and function, 711–712
- structural determinants of activation, 492–494
- subunit genes, 469–470
 - central nervous system distribution, 471–473
- transmitter inactivation, 51–52
- transporters, 467–468
- Gamma glutamate analogs, kainate receptor orthosteric agonists, 378–379
- γ -glutamyl transpeptidase, nitric oxide nitrosylation, 747
- Ganaxolone, GABA_A receptor modulation, 515–516
- Gaseous signaling
 - carbon monoxide, 756–757
 - future research, 757
 - nitric oxide
 - basic principles, 743–744
 - neurodegeneration and, 754
 - nitrosothiol detection, 747–748
 - nitrosylation mechanism, 745–747
 - cell survival, 752
 - extracellular matrix, 751–752
 - gene transport, 750–751
 - ion channels, 748–749
 - Parkinson's disease and, 755–756
 - protein-protein interactions, 749–750
 - S nitrosylation physiology, 752–754
 - vesicular transport, 751
 - physiological role, 745
 - packaging of, 46
 - synthesis, 45
- Gastric acid secretion, muscarinic acetylcholine receptor deficiency, 175
- Gastrointestinal disorders, serotonin transporters and, 720
- Gating mechanisms, voltage-gated potassium channels, 626–627
- G $\beta\gamma$ signaling, to ion channels, 67–69
- Geller-Seifter conflict test, mGlu5 allosteric antagonists, 444
- Gene duplication, neuropeptides, 680–682
- Gene expression, neuropeptides, 685
- Gene splicing
 - neuropeptides, 685
 - plasma membrane transporter regulation, 725
- Gene therapy, vesicular transporters and, 723
- Genetic absence-epilepsy rats from Strasbourg (GAERS), GABA_B receptors, 595
- Genetic analysis
 - α_1 adrenergic receptors, 201–202
 - β -adrenergic receptor physiology, 211–213
 - bipolar disorder, 860
 - GABA_A receptor subunits, 469–470
 - neuropeptides, 678–679
 - knockout mice, 678
 - transgenic animals, 678

- norepinephrine transporter, 200–201
- Parkinson's disease and, 755–756
- Genetic linkage studies
 - BDNF in mood disorders, 803
 - GABA_B receptor targeting, 596–597
- Gene transcription, nitrosylation and, 750–751
- Genome sequencing, potassium channels, 621–623
- Genomics, neuropeptides and, 678–679
- Gephyrin, GABA_A receptor clusters, 478–480
- GHB (Xyrem), GABA_B receptor targeting, 592–593
- Giant depolarizing potentials, GABA transporters, 467–468
- GLAST knockout mice, plasma membrane glutamate transporters, 711
- Glial-derived neurotrophic factor (GDNF), intracellular signaling, 793–794
- Glial proliferation
 - antidepressant influences on, 807
 - stress and, 800
- GLT1 knockout mice, plasma membrane glutamate transporters, 711
- Glucagon-like peptides, neuropeptide inactivation, 687–688
- Glucagon secretion, muscarinic acetylcholine receptor deficiency, 174–175
- Glucocorticoids
 - in bipolar disorder patients, 861–862
 - neurogenesis regulation by, 830
 - stress influence on BDNF and, 796–797
 - stress response and, 694–695
- Glucose-related protein 78 (GRP78), in bipolar disorder patients, 870
- Glutamate
 - ionotropic receptors
 - allosteric potentiators and antagonists, 385–397
 - AMPA receptors, 370–371
 - allosteric antagonists, 395–396
 - allosteric potentiators, 390–395
 - orthosteric agonists, 376–377, 378–381
 - orthosteric antagonists, 377–378, 381–385
 - classification and background, 365–366
 - future research issues, 397–398
 - kainate receptors, 371–372
 - allosteric antagonists, 397
 - allosteric potentiators, 396
 - NMDA receptors, 367–370
 - allosteric antagonists, 386–390
 - allosteric potentiators, 385–386
 - orthosteric agonists, 373
 - orthosteric antagonists, 373–376
 - orthosteric pharmacological agents, 373–385
 - subtypes, 366–367
 - synthesis and storage, 366
- metabotropic receptors
 - allosteric potentiators and antagonists
 - mGlu1 antagonists, 436–437
 - mGlu2/3 antagonists, 439–440
 - mGlu5 antagonists, 443–445
 - mGlu7 antagonists, 445
 - mGlu1 potentiators, 435–436
 - mGlu2 potentiators, 437–439
 - mGlu4 potentiators, 440–441
 - mGlu5 potentiators, 441–443
 - background and classification, 421–422
 - future research issues, 445–446
 - orthosteric agents
 - group III selective agonists, 431–433
 - group III selective antagonists, 433–435
 - group II selective agonists, 426–429
 - group II selective antagonists, 429–431
 - group I selective agonists, 423–424
 - group I selective antagonists, 424–426
- neurotransmitter mechanisms, 53–54
 - plasma membrane neurotransmitter transporters
 - clinical relevance, 718–719
 - interacting proteins, 728
 - second messenger regulation, 727–728
 - structure and function, 708–711
 - vesicular transporters
 - clinical relevance, 723
 - structure and function, 716
- L-Glutamic acid decarboxylase (GAD), GABA catalysis, 466
- Glutamine neurotransmitter transporter, structure and function, 715
- Glutamatergic signaling, in bipolar disorder patients, 862–863
- γ -D-Glutamylaminomethylsulfonic acid (GAMS)

- kainate receptor orthosteric antagonists, 385
- Glycine
 - binding site agonists, NMDA allosteric potentiators, 385–386
 - competitive site antagonists, NMDA receptors, 386–389
 - early research on, 32
 - neurotransmitter transporters
 - clinical relevance, 722–723
 - structure and function, 712
- Glycogen breakdown, second messengers and, 31–32
- Glycogen synthase kinase-3 (GSK-3), lithium mechanism in bipolar disorder patients, 864–869
- Glycosylation, plasma membrane transporter regulation, 725–726
- Golgi-specific DHHC zinc finger protein (GODZ), GABA_A receptor trafficking, 480
- Gonadotropin-releasing hormone (GnRH), neuropeptides and, 676
- G protein
 - in bipolar disorder patients, 861–862
 - muscarinic acetylcholine receptors, selectivity, 157–158
 - signal transducers, 60–66
 - cycle regulations, 64–65
 - disease and, 67
 - heterotrimeric protein structure, 60–63
 - RGS proteins, 65–66
 - small proteins, 63–64
- G-protein-activated inwardly rectifying K⁺ (GIRK) channel
 - signaling mechanisms, 67–69
 - structure and function, 635
- G-protein-coupled receptors (GPCRs). *See also* Adrenergic receptors
 - chemical release, 48–50
 - dopamine, 225
 - dopamine signal transducers, 230–232
 - GABA_B molecular determinants, 579–580
 - GABA_B receptors, dependent and independent effects, 580–581
 - glutamate neurotransmitters, 53–54
 - ion channel modulation, 66–71
 - downstream signaling molecules, 69–70
 - Gβγ signaling, 67–69
 - protein-protein interactions, 70–71
 - muscarinic acetylcholine receptors
 - basic properties, 155–157
 - downregulation, 163–164
 - internalization, 162–163
 - RGS proteins, 158
 - uncoupling, 162
 - muscarinic acetylcholine receptors and, 149–151
 - neuropeptide receptors, 689–690
- G-protein receptor kinases (GRKs)
 - α₂-adrenergic receptor regulation, 205–206
 - D₁ receptor subfamily, 225–227
 - muscarinic acetylcholine receptor phosphorylation, 164–166
 - phosphorylation, 90–92
 - RGS protein function, 65–66
- Growth factors, intercellular signaling, 44–45, 46
- GTPase-activating proteins (GAPs), cycle regulation, 64–65
- Guanine nucleotide exchange factors (GEFs), small G proteins, 63–64
- Guanosine diphosphate (GDP)
 - dopamine signal transducers, 231–232
 - heterotrimeric G protein binding, 60–73
- Guanosine triphosphate (GTP), heterotrimeric G protein binding, 60–73
- Guanylate cyclase (cGMP) receptors
 - neuropeptides, 691
 - nitric oxide gaseous signaling, nitrosylation, 745–747
- Guanylyl cyclases (GCs), cyclic nucleotide second messenger signaling, 78–80
- Haloperidol-induced catalepsy, muscarinic acetylcholine receptor deficiency, 172
- Heart function, muscarinic acetylcholine receptor deficiency, 175
- HERG gene
 - KCNH potassium channels and, 633–634
 - voltage-gated potassium channels, 623
- 3-Heteroaryl-5,6bis(aryl)-1-methyl-2-pyridone, GABA_A α₃ subunit pharmacology, 488
- Heteromeric structure
 - GABA_B receptors, 575–578
 - nicotinic acetylcholine receptors, 112–113, 116–125
- Hetero-oligomeric receptors, GABA_A receptors, subunit subtype composition, 476–477

- Heteroreceptors, muscarinic acetylcholine receptor deficiency, 176
- Heterotrimeric G proteins, structure and function, 60–63
- Hexamethonium blockers, nicotinic acetylcholine receptors, 121
- Hippocampal system
 - anatomy and physiology, 823–824
 - antidepressants and neurogenesis in
 - adult neurogenesis stages, 827–828
 - behavioral and animal models, 833
 - depression physiology and, 823–824, 826–827
 - future research on, 833
 - monoamines and, 822–823
 - research background, 821–822
 - serotonin/norepinephrine regulation, 831–833
 - stress and monoamine hypotheses, 825–826
 - stress hormone neurogenesis regulation, 829–830
 - synapses
 - 5-HT₇ receptor family, 276
 - mGlu5 allosteric potentiators, 441–442
 - muscarinic acetylcholine receptor
 - deficiency and suppression of, 172
 - neurotrophins and, stress and neurogenesis in, 798–799
 - vesicular transporters and, 723
- Hispidulin, GABA_A subunit pharmacology, allosteric ligand modulation, 506
- Histamine
 - in brain
 - biosynthesis, 305–306
 - early history, 300–301
 - inactivation, 306–307
 - metabolism, 305–307
 - molecular pharmacology, receptor
 - subtypes, 307–317
 - H₁ receptor, 307–312
 - H₂ receptor, 312–314
 - H₃ receptor, 314–316
 - H₄ receptor, 316
 - NMDA receptor interaction, 316–317
 - neuron activity and control, 317–322
 - electrophysiology, 317
 - pharmacological changes, 321–322
 - in vitro modulation, 317–319
 - in vivo changes, 319–321
 - neuronal organization, 301–305
 - afferents, 303–305
 - histaminergic pathways, 303
 - perikarya, 301–303
 - neuron physiology, 322–326
 - arousal, 322–324
 - cognitive functions, 324
 - nociception, 326
 - pituitary hormone secretion, 324–325
 - satiation, 325–326
 - seizures, 326
 - neuropsychiatric disease, 327–329
 - Alzheimer's disease, 327–328
 - anxiety/ADHD, 329
 - Parkinson's disease, 328
 - schizophrenia and antipsychotic actions, 327
 - early research on, 16–17
- Histamine *N*-methyltransferase (HMT), histamine inactivation, 306–307
- Histaminergic pathways, structure and function, 303
- Histaminergic perikarya, structure and function, 301–303
- L-Histidine decarboxylase (HDC)
 - histamine neuron physiology, arousal mechanisms, 322–324
 - histaminergic perikarya and, 301–303
- Histochemistry, neuropeptide identification, 674
- Homologous residues, GABA_A receptor
 - activation, channel gating binding, 497
- Homooligomeric receptors, GABA_A receptors, 476
- H₁ receptor
 - arousal mechanisms, 323–324
 - molecular pharmacology, 307–312
 - brain tissue responses, 309–311
 - distribution, 311–312
 - signaling mechanisms, 309
 - structure and properties, 307–309
- H₂ receptor, molecular pharmacology, 312–314
- H₃ receptor
 - cognitive functions, 324
 - histaminergic neuron activity, 321–322
 - molecular pharmacology, 314–316
- H₄ receptor, molecular pharmacology, 316
- 5-HT₇ receptor family, structure and function, 275–276

- 5-HT₁ receptor family. *See also* Serotonin systems
 classification, 263–264
 5-HT_{1A} receptors, 263, 266–267
 5-HT_{1B} and 5-HT_{1D} receptors, 267–268
 5-HT_{1E} receptors, 268–269
 5-HT_{1F} receptors, 269
 5-HT₂ receptor family, 269–273
 5-HT_{2A} receptors, 270–271
 5-HT_{2B} receptors, 271
 5-HT_{2C} receptors, 271–272
 5-HT₃ receptor family, structure and function, 273
 5-HT₄ receptor family, structure and function, 273–274
 5-HT₅ receptor family, structure and function, 274–275
 5-HT₆ receptor family, structure and function, 275
 Huntington's disease, H₁ receptor distribution, 313–314
 7-Hydroxyiminocyclopropan[*b*]chromene-1 α -carboxylic acid, mGlu1 allosteric antagonists, 436–437
 Hydroxylation, dopamine synthesis, 54–55
 5-Hydroxytryptamine (5-HT). *See also* 5-HT₁ receptor family; Serotonin systems
 Hyperkalemic periodic paralysis (HyperPP), voltage-gated sodium ion channels and, 641–642
 Hyperpolarization-activated channels (HCN), structure and function, 649–650
 Hypertension therapy, voltage-gated calcium channels, Ca_v1 family, 648
 Hypothalamic-pituitary-adrenal (HPA) axis
 hippocampal neurogenesis and depression and, 827
 stress response and, 694–695
 Hypothalamic-pituitary pathways, neuropeptides, 692
 Hypothalamic release-stimulating/release-inhibiting actions, neuropeptides, 691
 Hypothalamus
 histamine neuron physiology
 arousal mechanisms, 323–324
 satiation effects, 325–326
 neuropeptide control, 691–692
 Hypothermia, muscarinic acetylcholine receptor deficiency, 170–171
 Hypoxia, S nitrosylation physiology and, 753–754
 Ifenprodil, NMDA receptor allosteric antagonists, 390
 Imidazenil, GABA_A subunit pharmacology, allosteric ligand modulation, 504–506
 Iminodibenzyl derivatives, early research on, 30
 Imipramine, early research on, 30–31
 Immediate early genes, neuropeptide identification, 673
 Immunocytochemistry, neuropeptide identification, 673
 Immunosuppression, intermediate-conductance calcium-activated potassium channels, 631
 Inactivation
 histamines, 306–307
 neuropeptides, 687–688
 extracellular inactivation, 687–688
 neurotransmitters, 50–52
 of nicotinic acetylcholine receptor, 125–127
 potassium ion channels, 627
 Inhibitory postsynaptic potential (IPSP)
 early research on, 29
 neuropeptides and, 676–677
INO1 gene, lithium mechanism in bipolar disorder and, 869
 Inositol depletion, lithium mechanism in bipolar disorder and, 869
 In situ hybridization, neuropeptide identification, 674
 Insulin-like growth factor (IGF-1)
 depression models and, 809
 intracellular signaling, 763
 Insulin secretion
 β -adrenergic receptor physiology, 212–213
 GABA_A receptor trafficking, 480
 muscarinic acetylcholine receptor deficiency, 174–175
 neurotrophic factors and, 763
 Interacting proteins
 GABA_B receptor modulation, 585–587
 G-protein-coupled receptors, 70–71

- plasma membrane transporter regulation, 728
- voltage-gated potassium channels, 627–628
- Intercellular signaling, synaptic transmission
 - basic principles, 40–41
 - classical transmitters, 45–46
 - dopamine neurotransmitters, 54–56
 - endocannabinoids, purines, and gaseous transmitters, 46
 - glutamate neurotransmitters, 52–54
 - peptide transmitters and growth factors, 46
 - postsynaptic receptors, 48–50
 - presynaptic receptors, 50–51
 - synaptic release, 46–48
 - transmitter inactivation, 51–52
 - transmitter packaging, 45–46
 - transmitter synthesis, 41–45
 - amine transmitters, 42–44
 - endocannabinoids, 45
 - gaseous transmitters, 45
 - neuropeptides, neurotrophins, and growth factors, 44–45
 - vesicle-dependent release, 46–47
 - vesicle-independent release, 47–48
- Interleukin-1 β (IL- β), stress response and, 797
- Intermediate-conductance calcium-activated potassium channels (IK channels), structure and function, 631
- Internalization, muscarinic acetylcholine receptors, 162–163
- Intracellular signaling
 - GABA_B receptor modulation, 585–586
 - neuropeptide degradation, 687
 - neurotrophic factors, 791–794
 - fibroblast growth factor, 793
 - insulin/insulin-like growth factor, 793
 - nerve growth factor family, 791–792
 - transforming growth factor-beta, 793–794
 - vascular endothelial growth factor, 792–793
- synaptic transmission
 - basic principles, 59–60
 - calcium channel calmodulin mediator, 85–87
 - calcium channel signaling molecules, 83–85
 - cyclic nucleotide second messengers, 72–81
 - adenylyl cyclase, 72–77
 - cAMP targets, 75, 78
 - cGMP cellular targets, 80–81
 - guanylyl cyclase, 78–80
 - DAG activation of protein kinase C, 85
 - GPCR-G protein ion channel
 - modulation, 66–71
 - downstream signaling molecules, 69–70
 - G $\beta\gamma$ signaling, 67–69
 - protein-protein interactions, 70–71
- G protein signal transducers, 60–66
 - cycle regulations, 64–65
 - disease and, 67
 - heterotrimeric protein structure, 60–63
 - RGS proteins, 65–66
 - small proteins, 63–64
- IP₃ and phosphoinositide signaling molecules, 81–83
- protein phosphorylation, 87–96
 - G-protein-coupled receptor kinases, 90–92
 - mitogen-activated protein kinase, 89–90
 - protein tyrosine kinase, 87–89
 - protein tyrosines phosphatases, 92–94
 - serine/threonine phosphatases, 94–96
- Introns, H₃ receptor, 314–315
- Inverse agonism, GABA_A receptor
 - activation, allosteric ligand
 - modulation, 500–506
- In vitro modulation, histaminergic neuron activity, 317–319
- In vivo modulation
 - in bipolar disorder patients, 863
 - GABA_B deficient mice, 587–588
 - histaminergic neuron activity, 319–322
- Inwardly rectifying potassium, structure and function, 634–635
- Ion channels
 - early research on, 25
 - GABA_A receptor activation, channel pore complex, 497–498
 - GABA_B receptor and, 469
 - G-protein-coupled receptor modulation, 66–71
 - downstream signaling molecules, 69–70
 - G $\beta\gamma$ signaling, 67–69
 - protein-protein interactions, 70–71
- muscarinic acetylcholine receptor
 - modulation, 160–162
- nitrosylation in, 748–759

- Ionotropic channels
 - chemical release, 48–50
 - glutamate receptors
 - allosteric potentiators and antagonists, 385–397
 - AMPA receptors, 370–371
 - allosteric antagonists, 395–396
 - allosteric potentiators, 390–395
 - orthosteric agonists, 376–377, 378–381
 - orthosteric antagonists, 377–378, 381–385
 - classification and background, 365–366
 - future research issues, 397–398
 - kainate receptors, 371–372
 - allosteric antagonists, 397
 - allosteric potentiators, 396
 - NMDA receptors, 367–370
 - allosteric antagonists, 386–390
 - allosteric potentiators, 385–386
 - orthosteric agonists, 373
 - orthosteric antagonists, 373–376
 - orthosteric pharmacological agents, 373–385
 - subtypes, 366–367
 - synthesis and storage, 366
- IP₃ signaling molecules, intracellular signaling, 81–83
- Ischemia. *See* Stroke therapy
- Isoquinolin-pyrimidines, NMDA receptor allosteric antagonists, 390
- Iso receptors, neuropeptides, 689
- JC virus, 5-HT_{2A} receptors and, 271
- Jervall-Lange-Nielsen syndrome, KCNQ channels and, 632–633
- K36, GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
- Kainate receptors
 - allosteric antagonists, 397
 - allosteric potentiators, 396
 - orthosteric agonists, 378–381
 - orthosteric antagonists, 381–385
 - structure and function, 371–372
- K⁺ channel interacting protein (KChIPs), voltage-gated potassium channels, 628
- KCNE proteins, voltage-gated potassium channels, 628
- KCNH potassium channels
 - clinical conditions and, 633–634
 - structure and function, 623
- KCNQ channels
 - clinical applications, 632–633
 - structure and function, 623
- KcsA channel, gating mechanism, 626–627
- Ketamine
 - NMDA receptor allosteric antagonists, 389–390
 - NMDA receptors, 368–370
- Knockout (KO) mice
 - GABA_A subunit pharmacology, α subunits, α_1 subunit, 484, 488
 - GABA_B deficient mice, 587–588
 - neuropeptide gene targeting in, 678
 - plasma membrane glutamate transporters, 711
- Kymarenic acid, kainate receptor orthosteric antagonists, 385
- Kynuremic acid, NMDA receptor allosteric antagonists, 387
- L-655,708, GABA_A α_5 subunit pharmacology, 489
- L-838,417, GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
- Large-conductance calcium-activated potassium channels (BK channels), structure and function, 630–631
- Large dense-core vesicles (LDCVs), neuropeptide electrophysiology, 675–676
- Learning function, muscarinic acetylcholine receptor deficiency, 168–169
- Leucine binding protein (LBP), GABA_B receptor sites, 578–579
- Leucine residues, GABA_A receptor activation, channel pore complex, 497–498
- Leukotriene D4 (LTDR), mGlu2 allosteric potentiators, 439
- Ligand binding core (LBC)
 - AMPA receptor allosteric potentiators, 393–395
 - AMPA receptor orthosteric agonists, 377
 - AMPA receptors, 370–371
- Ligand binding domain (LBD)
 - dopamine, 233–234
 - GABA_A receptors

- agonist binding site architecture, 494–495
- allosteric ligand modulation, 499–506
- assembly, 477–478
- GABA_B receptor sites, 578–579
- muscarinic acetylcholine receptors (mAChRs), 152–154
 - agonists, 152
 - allosteric ligands, 153–154
 - antagonists, 152–153
 - clinical applications, 154
 - serotonin receptor families, 263–267
- Ligand-gated ion channels
 - GABA_A receptors, desensitization and deactivation, 498–499
 - glutamate neurotransmitters, 53–54
 - 5-HT₃ receptor family and, 273
- Lithium, mechanism in bipolar disorder patients, 864–869
- Lobeline, vesicular transporters and, 723
- Localization studies
 - H₂ receptor, 313–314
 - H₁ receptor distribution, 311–312
 - H₃ receptors, 316
- Locomotor activity
 - GABA_A α_6 subunit pharmacology, 489–490
 - mGlu5 allosteric potentiators, 442–443
 - mGlu II selective orthosteric antagonists, 431
 - muscarinic acetylcholine receptor deficiency, 169–170
- Long-QT syndrome
 - KCNQ channels and, 632–633
 - voltage-gated sodium ion channels and, 642
- Long-term potentiation (LTP), adult neurogenesis and, 829
- L*-4-phosphono-2-aminobutyric acid (*L*-AP4), mGlu III selective orthosteric agonists, 433–435
- Luteinizing hormone releasing hormone (LHRH), histaminergic neuron activity, 325
- LY34195, mGlu II selective orthosteric antagonists, 430–431
- LY354740, mGlu II selective orthosteric agonists, 426–428
- LY367385, mGlu I selective orthosteric antagonists, 425–426
- LY392098, AMPA receptor allosteric potentiators, 390–391
- LY487379, mGlu2 allosteric potentiators, 437–439
- LY520303, mGlu II selective orthosteric agonists, 426–427
- LY4464333, mGlu II selective orthosteric agonists, 426–427
- LY379268/LY389795, mGlu II selective orthosteric agonists, 428–429
- Magnesium ions
 - NMDA receptor allosteric antagonists, 387–389
 - second messengers and, 31–32
- Magnocellular system,
 - hypothalamic-pituitary pathways, 692
- Major depressive disorder (MDD). *See* Depression
- Marine products, kainate receptor orthosteric agonists, 379–381
- Matrix metalloproteinases (MMPs), nitrosylation and, 751–752
- MCG-1, mGlu II selective orthosteric agonists, 426–427
- McN-A-343 muscarinic agonist,
 - cardiovascular effects, 176
- Mecamylamine, nicotinic acetylcholine receptors, 121
- Melanocortin, neuropeptide receptors, 690
- Melanocyte-stimulating hormone (MSH), neuropeptides and, 677–678
- Melatonin
 - depression and levels of
 - in menopause, 849–850
 - during menstrual cycle, 844–845
 - postpartum depression, 848
 - in pregnancy, 846–847
 - reproductive-cycle depression and
 - levels of, in menstrual cycle, 844–845
- Membrane-associated guanylate kinase (MAGUK) family, voltage-gated calcium channels, β subunits, 645–646
- Membrane potentials
 - early research on, 25–27
 - inward rectification, with potassium channels, 634–635
- Memory function, muscarinic acetylcholine receptor deficiency, 168–169

- Menopause, neuroendocrine abnormalities and depression in, 849–851
- Menstrual cycle depression, neuroendocrine abnormalities, 844–846, 851
- cortisol, 845
- melatonin, 844–845
- prolactin, 846
- thyroid-stimulating hormone, 845
- Messenger RNA (mRNA)
- D₁ receptor expression, 226–227
- D₂ receptor expression, 229–230
- large-conductance calcium-activated potassium channels, structure and function, 630–631
- mGlu group I selective orthosteric agonists, 423
- Metabotropic receptors
- γ-amino butyric acid (GABA) receptors
- agonists and competitive antagonists, 589–590
- disease and, 592–597
- anxiety and depression, 594–595
- drug addiction, 593–594
- epilepsy, 595
- gene linkage studies, 596–597
- nociception, 595–596
- therapeutic targeting, GABA_B receptors, 592–593
- tumor cell growth and migration, 596
- effector systems, 580–583
- adenylate cyclase, 582–583
- calcium channels, 581–582
- G-protein-dependent/independent GABA_B effects, 580–581
- MAPKs, 583
- potassium channels, 582
- endogenous GABA_B ligands, 588–589
- GABA_B deficient mice, 587–588
- GABA_B novel compounds, 590–592
- GABA_B receptor modulation, 584–586
- extracellular calcium, 584
- interacting proteins, 585–587
- phosphorylation and desensitization, 584–585
- research background, 570–571
- structural properties, 571–580
- allosteric interactions, 580
- expression cloning, 571–572
- G-protein coupling determinants, 579–580
- ligand binding sites, 578–579
- molecular subtypes, 572–575
- surface trafficking and heteromerization, 575–578
- glutamate receptors
- allosteric potentiators and antagonists
- mGlu1 antagonists, 436–437
- mGlu2/3 antagonists, 439–440
- mGlu5 antagonists, 443–445
- mGlu7 antagonists, 445
- mGlu1 potentiators, 435–436
- mGlu2 potentiators, 437–439
- mGlu4 potentiators, 440–441
- mGlu5 potentiators, 441–443
- background and classification, 421–422
- future research issues, 445–446
- orthosteric agents
- group III selective agonists, 431–433
- group III selective antagonists, 433–435
- group II selective agonists, 426–429
- group II selective antagonists, 429–431
- group I selective agonists, 423–424
- group I selective antagonists, 424–426
- Metal ions, voltage-gated calcium channel blockers, 647
- Methamphetamine, histaminergic neuron activity, 327
- 3-Methoxy-4-hydroxyphenol-glycol (MHPG), norepinephrine neurochemistry, 198–199
- 3-(2-Methoxy-phenyl)-5-methyl-6-phenyl-5*H*-isoxazolol [4,5-*c*]pyridin-4-one, mGlu7 allosteric antagonists, 445
- (2*S*,1'*S*,2'*S*)-2-Methyl-2(2'-carboxycyclopropyl)glycine (MCCG), mGlu II selective orthosteric antagonists, 429–430
- S-α-Methyl-4-carboxyphenylglycine (S-MCPG), mGlu I selective orthosteric antagonists, 425
- Methyl methanethiosulfonate, nitrosothiol detection, 748
- 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Parkinson's disease and, 755
- (*R,S*)-α-Methyl-4-phosphonophenylglycine (MPPG), mGlu III selective orthosteric agonists, 434–435
- MGS0028, mGlu II selective orthosteric agonists, 429

- MGS0039, mGlu II selective orthosteric antagonists, 431
- Microdomains, dopamine, 233–234
- Miniature end-plate potentials (MEPPs), early research on, 26–27
- Mitogen-activated protein kinase (MAPK)
 α_1 adrenergic receptor signal transduction pathways, 203
 GABA_B receptors, effector systems, 583
 lithium mechanism in bipolar disorder patients, 867–869
 muscarinic acetylcholine receptor modulation, 159
 phosphorylation, 89–90
 receptor-like PTPs, 92–94
 small G proteins, 63–64
- Monoamine oxidase B (MAOB), Parkinson's disease and, 755
- Monoamine oxidase (MAO)
 α_2 -adrenergic receptor regulation, 205–206
 dopamine inhibition, 223–224
 mGlu5 allosteric antagonists, 443–444
 norepinephrine neurochemistry, 198–199
 serotonin degradation and reuptake, 261–262
- Monoamines
 antidepressants and, hippocampal neurogenesis and, 822–823
 neurotransmitter inactivation, 51–52
 plasma membrane transporters, 719–720
 stress and depression convergence in, 825–826
 vesicular monoamine neurotransmitter transporters, 716–717, 723
- Mood disorders. *See also* specific disorders, e.g. Depression
- neurotrophic factors
 antidepressants
 BDNF expression and, 804–806
 neurogenesis and, 806–808
 behavioral effects, 808–809
 brain-derived neurotrophic factor genetics, 803
 depressed patients, 800–802
 structural and cellular alterations, 802–803
 intracellular signaling cascades, 791–794
 fibroblast growth factor, 793
 insulin/insulin-like growth factor, 793
 nerve growth factor family, 791–792
 transforming growth factor-beta, 793–794
 vascular endothelial growth factor, 792–793
 research background, 790
 stress and, 794–797
 adrenal glucocorticoids, 796–797
 adult neurogenesis and morphology, 797–800
 hippocampus, 798
 alterations in, 794–795
 brain-derived neurotrophic factor, 794–796
 cytokines, 797
 glial proliferation, 800
 serotonin receptors, 797
 vascular endothelial growth factor, 797
- Morphometric studies, depressed patients, neurotrophic factors and, 802–803
- Motor neuron diseases, plasma membrane glutamate transporter, 718–719
- MPEP
 mGlu4 allosteric antagonists, 440–441
 mGlu5 allosteric antagonists, 443–444
- MTEP, mGlu5 allosteric antagonists, 444
- MthK channel, gating mechanism, 626–627
- Multifocal leukoencephalopathy (PML), 5-HT_{2A} receptors and, 271
- Multiple sclerosis (MS)
 nitric oxide physiology, 745
 voltage-gated sodium ion channels, 640–641
- Muscarinic acetylcholine receptors (mAChRs)
 activation mechanisms, 155
 dimerization, 166–127
 distribution, 149
 G-protein coupling properties, 155–157
 RGS proteins, 158
 G protein selectivity, 157–158
 ion channels, 160–162
 ligand binding mechanisms, 152–154
 agonists, 152
 allosteric ligands, 153–154
 antagonists, 152–153
 clinical applications, 154
 MAPK pathways modulation, 159
 phenotypic mouse analysis, 167–177

- agonist-induced tremor and
 - hypothermia, 170–171
- amylase secretion, exocrine pancreas, 175
- analgesia, 170
- autoreceptors, 171
- cardiovascular system, 175–176
- cytolytic T cells, 177
- drug abuse effects, 173
- epileptic seizures, 169
- food intake stimulation, 171
- gastric acid secretion, 175
- inhibitory hippocampal synapse
 - suppression, 172
- learning and memory functions,
 - 168–169
- locomotor activity, 169–170
- nucleus accumbens, dopamine effects,
 - 172–173
- pancreatic islet insulin and glucagon
 - secretion, 174–175
- peripheral autoreceptors and
 - heteroreceptors, 176
- prepulse inhibition and
 - haloperidol-induced catalepsy, 172
- salivary secretion, 174
- skin functions, 177
- smooth muscle functions, 173–174
- striatal dopamine release modulation,
 - 171–172
- regulatory mechanisms, 162–166
 - downregulation, 163–164
 - internalizations, 162–163
 - phosphorylation, 164–166
 - resensitization, 166
 - uncoupling, 162
- research background, 148–149
- signaling pathways, 158–159
- structural features, 149–151
- Mutagenesis
 - GABA_A receptor activation
 - benzodiazepine binding sites, 506–507, 509–511
 - channel gating binding, 496–497
 - potassium channels, voltage activation,
 - 625–626
- Mutations, in G proteins, 66
- Myotonias, voltage-gated sodium ion
 - channels and, 641–642
- Myristoylated alanine rich C-kinase
 - substrate (MARCKS), lithium
 - mechanism in bipolar disorder
 - patients, 868–869
- National Institute for Medical Research,
 - establishment of, 17–18
- Natural products, AMPA receptor
 - orthosteric agonists, 376–377
- Negative modulators
 - GABA_A α_1 subunit pharmacology,
 - 488
 - GABA_A δ subunit pharmacology, 492
 - GABA_A γ subunit pharmacology, 491
 - GABA_A subunit pharmacology, 483
- Neodysiherbaine, kainate receptor
 - orthosteric agonists, 380
- Nerve growth factor (NGF) family,
 - receptors and kinases in, 791–792
- Nerve injury, voltage-gated sodium ion
 - channels, 640–641
- N*-ethylmaleimide-sensitive factor (NSF)
 - GABA_A receptor trafficking, 480
 - nitrosylation and, 751
- Neuroanatomy, bipolar disorder, 860–861
- Neurochemistry, bipolar disorder, 861–862
- Neurodegeneration, nitric oxide and, 754
- Neuroendocrine compounds
 - circadian studies of reproductive cycle
 - depression
 - cross-reproductive cycle analysis,
 - 851–853
 - menopause, 849–851
 - cortisol, 850
 - melatonin, 849–850
 - prolactin, 850–851
 - thyroid-stimulating hormone, 850
 - menstrual cycle, 844–846
 - cortisol, 845
 - melatonin, 844–845
 - prolactin, 846
 - thyroid-stimulating hormone, 845
 - postpartum depression, 848–849
 - cortisol, 848
 - estradiol, 849
 - melatonin, 848
 - prolactin, 848–849
 - thyroid-stimulating hormone, 848
 - pregnancy, 846–847
 - cortisol, 847
 - melatonin, 846–847
 - prolactin, 847
 - thyroid-stimulating hormone, 847
 - research background, 844
 - neuropeptide electrophysiology, 675–676
- Neurogenesis
 - adult stages of, 827–829

- antidepressant influences on, 806–808
- serotonin/norepinephrine regulation, 831–833
- stress and
 - hormone regulation, 829–830
 - neurotrophins and, 797–800
- Neurohypophyseal gene family,
 - neuropeptides and, 682–684
- Neurological disorders
 - GABA transporters and, 722
 - monoamine plasma membrane transporters, 719–720
- Neuromodulators, neuropeptides as, 676–677
- Neuromuscular transmission
 - early research on, 12–13
 - Eccles, Kuffler, and Katz research on, 22–23, 25–26
 - electric organ model of, 22
 - Kuffler's research on, 32–33
 - Loewi's experiments in, 18–19
 - Nachmansohn's research on, 23
 - Nobel Prize awarded for research on, 21–22
 - voltage-gated sodium ion channel trafficking, 638–639
- Neuronal nicotinic receptors
 - history of, 107–108
 - overview, 110–111
 - pharmacology of subtypes, 113–125
- Neuronal organization
 - adult neurogenesis and, 829
 - histaminergic systems, 301–305
 - afferents, 303–305
 - histaminergic pathways, 303
 - neuron activity and control, 317–322
 - electrophysiology, 317
 - pharmacological changes, 321–322
 - in vitro modulation, 317–319
 - in vivo changes, 319–321
 - perikarya, 301–303
 - KCNQ channel action on, 632–633
 - large-conductance calcium-activated
 - potassium channels, structure and function, 630–631
 - stress and, 800
 - voltage-gated sodium ion channels, 639–640
- "Neuron doctrine," basic principles of, 40–41
- Neuropeptides
 - administration, 693
 - basic principles, 670–671
 - behavioral techniques, 677–678
 - biosynthesis and processing, 685–687
 - preprohormones, 686
 - prohormones, 686
 - tissue specificity, 687
 - blood-brain barrier, 692–693
 - classification table, 672–673
 - electrophysiological techniques, 675–677
 - neurohormones, 675–676
 - neuromodulators, 676–677
 - neurotransmitters, 676
 - evolution, 679–685
 - gene duplication, 680–685
 - gene splicing, 685
 - neurohypophyseal family, 682–684
 - NPY family, 684
 - opioid family, 680–682
 - structural conservation, 679–680
 - extracellular inactivation, 687–688
 - future research issues, 695
 - gene isolation and expression, 685
 - genetic manipulations, 678–679
 - knockout mice, 678
 - transgenic animals, 678
 - genomics, 678–679
 - hypothalamic control of pituitary gland, 691–692
 - hypothalamic-pituitary pathways, 692
 - hypothalamic release-stimulating/
 - release-inhibiting actions, 691
 - identification, 671–674
 - inactivation, 687–688
 - intercellular signaling, 44–45
 - intracellular degradation, 687
 - isolation and characterization, 674
 - peptidomics, 675, 679
 - perfusion and tissue culture studies, 677
 - physiological/peptidomic/genomic techniques, 679
 - prolactin release, 691–692
 - receptors
 - agonists/antagonists, 675
 - arginine vasopressin, 689
 - cytokines, 691
 - downregulation and desensitization, 688–689
 - G-protein-coupled receptors, 690
 - guanylate cyclase (cGMP) receptors, 691

- isoreceptors, 689
- melanocortin, 690
- neuropeptide Y system, 689
- phospholipase-phosphatidylinositol-linked messengers, 690
- second-messenger systems and, 690–691
- structure and function, 688–690
- tachykinin, 689–690
- tyrosine kinase-coupled receptors, 691
- upregulation and sensitization, 689
- redundancy, 694
- site-directed mutagenesis, 679
- stress/neuronal response, 694–695
- time- and tissue-sensitive responses to, 693–694
- Neuropeptide Y system
 - gene duplication and divergence in, 684
 - in knockout mice, 678
 - neuropeptide receptors, 689
 - physiology, 679
- Neuropharmacology
 - Bovet's contributions to, 25
 - Forster's contributions in, 9–11
 - Gaskell and Langley's contributions to, 11–13
 - postwar trends in, 24–25
- Neuropsychiatric disease. *See also* Mood disorders
 - AMPA receptor allosteric potentiators, 394–395
 - GABA_A subunit pharmacology, allosteric ligand modulation, 505–506
 - histaminergic neuron activity, 321–322, 327–329
 - Alzheimer's disease, 327–328
 - Parkinson's disease, 328
 - schizophrenia and antipsychotics, 326
 - monoamine plasma membrane transporters, 719–720
 - plasma membrane glutamate transporter, 718–719
 - vesicular transporters and, 723
- Neurosteroids
 - GABA_A δ subunit pharmacology, 491–492
 - GABA_A modulation, 511–516
 - transmembrane domains, 518
- Neurotoxins
 - AMPA receptor orthosteric agonists, 376–377
 - sodium voltage-gated ion channels, 636
 - voltage-gated calcium ion channels, Ca_v2 family, 649
 - voltage-gated potassium channels, K_v subunits, structure and function, 630
 - voltage-gated sodium channels and, 642–643
- Neurotransmitters
 - biosynthesis of, 42–43
 - dopaminergic
 - attention-deficit hyperactivity disorder, 237–238
 - basic properties, 221–222
 - bipolar disorders, 237
 - chemistry and metabolism, 222–224
 - classification and molecular properties, 225–230
 - D₁ receptor subfamily, 225–227
 - D₂ receptor subfamily, 227–230
 - drug applications, 236–239
 - Parkinson's disease, 236
 - polymorphisms, splice variants and SNPs, 234–236
 - schizophrenia, 237
 - signal transduction pathways, 230–232
 - structure-affinity/structure-activity relationships, 232–234
 - substance abuse, 238
 - GABA_A receptor trafficking, 480
 - inactivation, 51–52
 - neuropeptide electrophysiology, 675–676
- transporters
 - clinical relevance, 718–723
 - dopamine, 721
 - GABA transporters, 721–722
 - glycine transporters, 722–723
 - monoamine transporters, 719–720
 - norepinephrine, 721
 - plasma membrane, 718–721
 - serotonin, 720
 - system interactions, 723
 - early research, 706–707
 - plasma membrane family, 707–715
 - choline, 714–715
 - clinical relevance, 718–723
 - dopamine, 712–713, 721
 - GABA transporters, 711–712, 721–722
 - glutamate transporters, 708–711, 718–719

- glutamine, 715
- glycine transporters, 712, 722–723
- monoamine transporters, 719–720
- norepinephrine, 713–714, 721
- regulation, 724–728
- serotonin, 714, 720
- sodium/chloride-dependent transporters, 711–714
- vesicular transporters, 715–718
 - acetylcholine and monoamine transporters, 716–718
 - clinical relevance, 723
 - glutamate transporters, 716
 - inhibitor amino acid transporters, 718
- voltage-gated calcium ion channels, Ca_v2 family, 648–649
- voltage-gated potassium channels, K_v subunits, structure and function, 628–630
- Neurotrophic factors
 - intercellular signaling, 44–45
 - mood disorders
 - antidepressants
 - BDNR expression and, 804–806
 - neurogenesis and, 806–808
 - behavioral effects, 808–809
 - brain-derived neurotrophic factor
 - genetics, 803
 - depressed patients, 800–802
 - structural and cellular alterations, 802–803
 - intracellular signaling cascades, 791–794
 - fibroblast growth factor, 793
 - insulin/insulin-like growth factor, 793
 - nerve growth factor family, 791–792
 - transforming growth factor-beta, 793–794
 - vascular endothelial growth factor, 792–793
 - research background, 790
 - stress and, 794–797
 - adrenal glucocorticoids, 796–797
 - adult neurogenesis and morphology, 797–800
 - hippocampus, 798
 - alterations in, 794–795
 - brain-derived neurotrophic factor, 794–796
 - cytokines, 797
 - glial proliferation, 800
 - serotonin receptors, 797
 - vascular endothelial growth factor, 797
- Neurotrophin-3 (NT-3), intracellular signaling, 791–792
- Neurotrophin-4 (NT-4), intracellular signaling, 791–792
- Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase channel, structure and function, 649–650
- Nicotine, early research on, 11–12
- Nicotinic acetylcholine receptor (nAChR)
 - action potential research and, 26–27
 - agonist-induced upregulation, 127–130
 - cloning of, 27–28
 - GABA_A receptor structure and, 475
 - heteromeric structures, 116–125
 - α₇nAChR subunit, 114–116
 - overview, 110–111
 - pharmacology of subtypes, 113–125
 - regulation of, 125–130
 - agonist-induced upregulation, 127–130
 - desensitization and inactivation, 125–127
 - structure of, 111–113
 - + TC actions and, 27
- Nicotinic acetylcholine receptors (nAChRs), history of, 107–110
- Nicotinic receptors, histaminergic neuron activity, 321–322
- Nitric oxide (NO), gaseous signaling
 - basic principles, 743–744
 - neurodegeneration and, 754
 - nitrosothiol detection, 747–748
 - nitrosylation mechanism, 745–747
 - cell survival, 752
 - extracellular matrix, 751–752
 - gene transport, 750–751
 - ion channels, 748–749
 - Parkinson's disease and, 755–756
 - protein-protein interactions, 749–750
 - S nitrosylation physiology, 752–754
 - vesicular transport, 751
 - physiological role, 745
- Nitric oxide synthases (NOSs), nitric oxide synthesis, 743–744
- S-Nitrosocysteineyl glycine (CGSNO), nitric oxide nitrosylation, 747
- S-Nitrosoglutathione (GSNO), nitric oxide nitrosylation, 747

- S*-Nitrosogluthathione reductase (GSNOR),
nitric oxide nitrosylation, 747
- Nitrosothiols
nitric oxide gaseous signaling, 747–748
S nitrosylation physiology and, 753–754
- Nitrosylation, nitric oxide gaseous signaling,
745–747
cell survival, 752
extracellular matrix, 751–752
gene transport, 750–751
ion channels, 748–749
Parkinson's disease and, 755–756
protein-protein interactions, 749–750
S nitrosylation physiology, 752–754
vesicular transport, 751
- NMDA (*N*-methyl-D-aspartic acid)
receptors
allosteric antagonists, 386–390
allosteric potentiators, 385–396
in bipolar disorder patients, 863
lithium mechanisms, 868–869
GABA_B receptor trafficking and
heteromization, 575–576
histamine receptor interaction, 316–317
mGlu5 allosteric antagonists, 443–444
mGlu group I selective orthosteric
agonists, 424
nitric oxide physiology, 745
nitrosylation, 748–749
orthosteric agonists, 373
orthosteric antagonists, 373–376
stress and neurogenesis and, 799–800
structure and function, 367–370
voltage-gated potassium channels, K_V
subunits, structure and function,
629–630
- Noceptin, histaminergic neuron activity,
319
- Nociception
GABA_B receptor targeting, 595–596
histamine neuron physiology, 326
voltage-gated calcium ion channels, Ca_v2
family, 649
- Norepinephrine (noradrenaline)
 α -adrenergic receptors, α_2 -adrenergic
receptor, 204–209
 β -adrenergic receptor activation, 209–210
antidepressant influences on, hippocampal
neurogenesis and, monoamines,
822–823
basic properties, 193
dopamine synthesis, 56, 223–224
early research on, 29–30
historical perspective, 194–196
mood disorders and, basic principles, 790
neurochemistry, 198–199
neurogenesis regulation, 831–833
neurotransmitter transporters
chronic substrate treatment, 726–727
clinical relevance, 721
structure and function, 713–714
physiology, 197–198
- Norepinephrine transporter (NET)
agent selectivity, 199–200
general characteristics, 199–200
genetic variations, 200–201
- NS3763, kainate receptor allosteric
antagonists, 397
- Nuclear receptors, chemical release, 48–50
- Nucleus accumbens, muscarinic
acetylcholine receptor deficiency,
dopamine efflux, 172–173
- Nucleus basalis magnocellularis, H₁
receptors in, 311
- Null modulators, GABA_A subunit
pharmacology, 483
- Obsessive compulsive disorder (OCD),
serotonin transporters and, 720
- Ocinaplon, GABA_A α_2 subunit
pharmacology, 488
allosteric ligand modulation, 505–506
- Oocyte expression systems, heteromeric
nicotinic acetylcholine, 117–119
- Opioid family, neuropeptide gene
duplication in, 680–682
- Opioid receptor agonists, histaminergic
neuron activity, 321–322
- Orexins, histaminergic neuron activity,
318–319, 320–322
- Organic cation transporters (OCT),
histamine inactivation, 307
- Orthostatic intolerance, norepinephrine
transporters and, 721
- Orthosteric agents
ionotropic glutamate receptors,
373–385
AMPA receptor agonists, 376–377
AMPA receptor antagonists, 377–378
kainate receptor agonists, 378–381
kainate receptor antagonists, 381–385
NMDA receptor agonists, 373
NMDA receptor antagonists, 373–376
metabotropic glutamate receptors

- group III selective agonists, 431–433
- group III selective antagonists, 433–435
- group II selective agonists, 426–429
- group II selective antagonists, 429–431
- group I selective agonists, 423–424
- group I selective antagonists, 424–426
- Oxine derivatives, kainate receptor orthosteric antagonists, 381–382
- 2-Oxoglutarate (2-OG), plasma membrane glutamate transporters, 711
- Oxygen
 - nitric oxide gaseous signaling, nitrosylation, 746–747
 - S nitrosylation physiology and, 752–754
- Oxyhemoglobin, S nitrosylation physiology and, 752–754
- Oxytocin, neuropeptides and, 682–684
- Pain management
 - mGlu1 allosteric antagonists, 437
 - voltage-gated calcium ion channels, Ca_v2 family, 649
- Pancreatic islets, muscarinic acetylcholine receptor deficiency, 174–175
- Paralytic disorders, voltage-gated sodium ion channels and, 641–642
- Parkinson's disease
 - dopaminergic drugs, 236
 - dopamine transporters and, 721
 - histaminergic neuron activity, 328
 - nitric oxide and, 754–755
 - nitrosylation and, 755–756
 - plasma membrane glutamate transporter, 719
- Parvicellular system, hypothalamic-pituitary pathways, 692
- Peptide toxins
 - large-conductance calcium-activated potassium channels, 631
 - small-conductance calcium-activated potassium channels, 632
 - voltage-gated potassium channels, K_v subunits, structure and function, 630
- Peptide transmitters, intercellular signaling, 46
- Peptide transport systems, blood-brain barrier neuropeptides and, 692–693
- Peptidomics, neuropeptides, 675, 679
- Perfusion and tissue culture studies, neuropeptides, 677
- Periodic paralysis, voltage-gated sodium ion channels and, 641–642
- Peripheral nervous system
 - in bipolar disorder patients, 863–864
 - kainate receptors, 372
 - voltage-gated sodium ion channels, 640
- Peroxisomal proliferation activating receptors (PPARs), mGlu2 allosteric potentiators, 439
- Pharmacology, early research in, 11
- PHCCC, mGlu4 allosteric antagonists, 440–441
- Phencyclidine (PCP)
 - mGlu II selective orthosteric agonists, 428–429
 - mGlu II selective orthosteric antagonists, 430–431
 - NMDA receptors, 368–370
- Phenotypic mice, muscarinic acetylcholine receptor deficiency, 167–177
 - agonist-induced tremor and hypothermia, 170–171
 - amylase secretion, exocrine pancreas, 175
 - analgesia, 170
 - autoreceptors, 171
 - cardiovascular system, 175–176
 - cytolytic T cells, 177
 - drug abuse effects, 173
 - epileptic seizures, 169
 - food intake stimulation, 171
 - gastric acid secretion, 175
 - inhibitory hippocampal synapse suppression, 172
 - learning and memory functions, 168–169
 - locomotor activity, 169–170
 - nucleus accumbens, dopamine effects, 172–173
 - pancreatic islet insulin and glucagon secretion, 174–175
 - peripheral autoreceptors and heteroreceptors, 176
 - prepulse inhibition and haloperidol-induced catalepsy, 172
 - salivary secretion, 174
 - skin functions, 177
 - smooth muscle functions, 173–174
 - striatal dopamine release modulation, 171–172

- Phenyl-terazoyl acetophenone, mGlu2
allosteric potentiators, 439
- Phosphatase and tensin homolog (PTEN),
receptor-like PTPs, 94
- Phosphoinositide signaling molecules,
intracellular signaling, 81–83
- Phospholipase-phosphatidylinositol- linked
messengers, neuropeptide
receptors, 690
- Phosphorylase, glycogen breakdown, 31–32
- Phosphorylation
cyclic nucleotide second messenger
signaling, guanylyl cyclases, 78–80
GABA_A receptor modulation, 481–482
GABA_B receptors, 584–585
muscarinic acetylcholine receptors,
164–166
of proteins, intracellular signaling,
synaptic transmission, 87–96
G-protein-coupled receptor kinases,
90–92
mitogen-activated protein kinase, 89–90
protein tyrosine kinase, 87–89
protein tyrosines phosphatases, 92–94
serine/threonine phosphatases, 94–96
- Phylogenetic tree, potassium channels,
623–625
- Picrotoxin, GABA_A receptor inhibition,
468–469
- Pinocytosis, blood-brain barrier
neuropeptides and, 692–693
- Pituitary adenylate cyclase-activating
polypeptide (PACAP)
lithium mechanism in bipolar disorder
and, 867–869
neuropeptide hypothalamic control,
691–692
- Pituitary gland, neuropeptide hypothalamic
control of, 691–692
- Pituitary hormones, histamine neuron
physiology and secretion of,
324–325
- Plant products
GABA_A subunit pharmacology, allosteric
ligand modulation, 505–506
neuropharmacological research and role
of, 6
tubocurarine, small-conductance
calcium-activated potassium
channels, 632
- Plasma membrane neurotransmitters,
GABA transporters and, 467–468
- Plasma membrane neurotransmitter
transporter families, 707–715
choline, 714–715
clinical relevance, 718–723
dopamine, 721
GABA transporters, 721–722
glycine, 722–723
monoamine, 719–721
norepinephrine, 721
serotonin, 720
dopamine, 712–713, 721
GABA transporters, 711–712, 721–722
glutamate transporters, 708–711, 718–719
glutamine, 715
glycine transporters, 712, 722–723
monoamine transporters, 719–720
norepinephrine, 713–714, 721
regulation, 724–728
chronic substrate treatment, 726–727
multiple transcription initiation sites,
725–726
polymorphisms, 724–725
second messengers, 727–728
serotonin, 714, 720
sodium/chloride-dependent transporters,
711–714
dopamine transporters, 712–713
GABA transporters, 711–712
glycine transporters, 712
norepinephrine transporters, 713–714
serotonin transporters, 714
structure and function, 707–708
- Plic-1 protein, GABA_A receptor trafficking,
480
- Polyamines, NMDA receptor allosteric
antagonists, 389
- Polymorphisms
dopamine, 234–236
plasma membrane neurotransmitter
transporter regulation,
724–725
- Population growth, impact on science of,
7–9
- Positive modulators
GABA_A α_1 subunit pharmacology, 488
GABA_A δ subunit pharmacology, 492
GABA_A γ subunit pharmacology, 491
GABA_A receptor activation, allosteric
ligands, 503–506
GABA_A subunit pharmacology, 483
- Postmortem studies, depressed patients,
neurotrophic factors and, 800–803

- Postmortem studies, bipolar disorder, 860–861
- Postpartum depression, neuroendocrine abnormalities and, 848–849, 851
- Postsynaptic density protein (PSD), GABA_B receptor modulation, 586–587
- Postsynaptic receptors
 - GABA_A receptor distribution, 474–475
 - GABA_B receptor subtypes, 573–575
 - intercellular signaling, 48–50
- Posttraumatic stress disorder (PTSD), stress-monoamine convergence, 826
- Potassium channels
 - early research on, 25
 - GABA_B receptors, effector systems, 582
 - histaminergic neuron activity, 317–319
 - muscarinic acetylcholine receptor modulation, 160–161
 - voltage-gated ion channels, 621–635
 - calcium activation, 630–632
 - fast inactivation, 627
 - gating, 626–627
 - genetics, 619–620
 - interacting proteins, 627–628
 - inwardly-rectifying and two-P channels, 634–635
 - KCNH channels, 633–634
 - KCNQ family, 632–633
 - K_v subunits, 628–630
 - K_v subunits, structure and function, 628–630
 - physiology, disease and pharmacology, 628–634
 - selectivity, 623–625
 - voltage activation, 625–626
- Pregnancy, depression in, neuroendocrine abnormalities and, 846–847, 851
- Pregnanolone sulfate
 - GABA_A δ subunit pharmacology, 492
 - GABA_A receptor modulation, 515
- Premenstrual cycle depressive disorder (PMDD). *See* Menstrual cycle depression
- Preprohormones, neuropeptide biosynthesis and, 686
- Prepulse inhibition
 - mGlu5 allosteric potentiators, 443
 - muscarinic acetylcholine receptor deficiency, 172
- Presynaptic receptors
 - α_2 adrenergic receptor physiology, 208–209
 - GABA_A receptor distribution, 474–475
 - GABA_B receptor subtypes, 573–575
 - histaminergic neuron activity, 321–322
 - intercellular signaling, 50–51
- Prohormones, neuropeptide biosynthesis and, 686
- Prolactin release
 - depression and levels of
 - in menopause, 850–851
 - during menstrual cycle, 846
 - postpartum depression, 848–849
 - in pregnancy, 847
 - neuropeptides, 691–692
- Promoter regions, plasma membrane transporter regulation, 725
- Pro-opiomelanocortin (POMC)
 - gene organization and expression, 685
 - neuropeptide gene duplication, 680–682
- Propranolol, β -adrenergic receptor activation, 210
- Protein kinase A (PKA)
 - in bipolar disorder patients, 862
 - cyclic nucleotide second messenger signaling, cAMP cellular targeting, 75, 78
 - GABA_A receptor phosphorylation, 481–482
 - GABA_B receptor desensitization and phosphorylation, 584–585
- Protein kinase C (PKC)
 - amino acid neurotransmitters, 43–44
 - DAG activation, 85
 - GABA_A receptor phosphorylation, 481–482
 - GABA_B receptor desensitization and phosphorylation, 584–585
 - GABA transporters, 467–468
 - heterotrimeric G proteins, 62–63
 - H₁ receptor signaling, 309–311
 - muscarinic acetylcholine receptor phosphorylation, 164
- Protein-protein interactions. *See also* Interacting proteins
 - G-protein coupled receptors, 70–71
 - nitrosylation and, 749–750
- Protein tyrosine kinases (PTKs), phosphorylation, 87–89
- Protein tyrosine phosphatases (PTPs), phosphorylation, 92–94
- Pseudogenes, D₁ receptor expression, 227

- Purines, packaging of, 46
- Pyramidal neurons, 5-HT_{2A} receptors in, 270–271
- Pyrethroid insecticides, voltage-gated sodium ion channels and, 643
- Pyridoindole, GABA_A subunit pharmacology, allosteric ligand modulation, 504–506
- Pyrrolidinones, AMPA receptor allosteric potentiators, 390
- Quinidine, voltage-gated potassium channels, K_V subunits, structure and function, 630
- Quinoazolines, AMPA receptor allosteric antagonists, 395–396
- Quinoxalinediones, kainate receptor orthosteric antagonists, 383
- Quinoxalines, AMPA receptor orthosteric antagonists, 377
- Quisqualate, mGlu group I selective orthosteric agonists, 423
- Radioimmunoassay (RIA), neuropeptide identification, 673
- Receptor activity modifying proteins (RAMPs), GABA_B receptor modulation, 585–586
- Receptor for activated C kinase (RACK-1), GABA_A phosphorylation, 482
- Receptor-like PTPs (RPTPs), phosphorylation, 92–94
- Receptors, serotonin family, 262–264
- Receptor tyrosine kinases (RTKs), signaling pathways, 88–89
- Redundancy, of neuropeptides, 694
- Reelin mRNA, in bipolar disorder patients, 862
- Release-inhibiting factor, neuropeptide hypothalamic control, 691–692
- Release-stimulating hormones, neuropeptide hypothalamic control, 691–692
- Repolarization, voltage-gated potassium channels, K_V subunits, structure and function, 628–630
- Reproductive cycle depression, neuroendocrine studies
 - cross-reproductive cycle analysis, 851–853
 - future research issues, 852–853
 - menopause, 849–851
 - cortisol, 850
 - melatonin, 849–850
 - prolactin, 850–851
 - thyroid-stimulating hormone, 850
- menstrual cycle, 844–846
 - cortisol, 845
 - melatonin, 844–845
 - prolactin, 846
 - thyroid-stimulating hormone, 845
- postpartum depression, 848–849
 - cortisol, 848
 - estradiol, 849
 - melatonin, 848
 - prolactin, 848–849
 - thyroid-stimulating hormone, 848
- pregnancy, 846–847
 - cortisol, 847
 - melatonin, 846–847
 - prolactin, 847
 - thyroid-stimulating hormone, 847
- research background, 844
- Resensitization, muscarinic acetylcholine receptor phosphorylation, 166
- Reserpine, early research on, 30–31
- Resting membrane potentials, KCNH potassium channels, 633–634
- Reuptake mechanisms
 - dopamine synthesis, 55
 - serotonin, 261–262
- RGS proteins
 - muscarinic acetylcholine receptors, 158
 - structure and function, 65–66
- RNA editing, 5-HT_{2C} receptors and, 272–273
- Ro 15-4513, GABA_A α_1 subunit pharmacology, 488
- RO 67-7476/67-4853, mGlu1 allosteric potentiators, 435–436
- RO 718218, mGlu2/3 allosteric antagonists, 439–440
- Romano-Ward syndrome, KCNQ channels and, 632–633
- Rostral agranular insular cortex (RAIC), GABA_B receptor targeting, 596
- RSSR signaling, GABA_B receptor trafficking and heteromization, 575–578
- Ryanodine, ion channel nitrosylation, 748–749
- Salivary secretion, muscarinic acetylcholine receptor deficiency, 174
- Satiation, histaminergic neuron activity, 325–326

- Saxitoxin (STX), voltage-gated sodium ion channels and, 642–643
- Scaffolding proteins, voltage-gated potassium channels, 628
- Schizophrenia
- dopaminergic drugs, 237
 - histaminergic neuron activity, 327
 - 5-HT_{2A} receptors and, 270–271
- Scientific research
- Dale and Loewi's contributions in, 14–15
 - Forster's contributions in, 9–11
 - German universities and, 9
 - population growth and, 7–9
 - postwar trends in, 24–25
 - role of patronage in, 7–8
 - Wellcome's contributions to, 15
- SCL17 transporter family, 716
- Second-messenger systems
- early research on, 31–32
 - glutamate transporter regulation, 727–728
 - neuropeptide receptors, 690–691
- Sedative effects
- α_2 adrenergic receptor physiology, 207–208
 - GABA_A β subunit pharmacology, 490–491
 - GABA_A γ subunit pharmacology, 491
 - GABA_A receptor activation, allosteric ligand modulation, 503–506
- Seizure disorders. *See also* Epileptic seizures
- GABA transporters and, 722
 - histamine neuron physiology, 326
 - KCNQ channels and, 633
- Selectivity
- potassium channels, 623–625
 - sodium voltage-gated ion channels, 637
- Serine/threonine phosphatases, phosphorylation, 94–96
- Serotonergic neurons, model of, 264
- Serotonin (5-HT)
- anatomy, 260
 - antidepressant influences on, 806–808
 - hippocampal neurogenesis and, monoamines, 822–823
 - stress-monoamine convergence, 825–826
 - basic properties, 257–259
 - degradation and reuptake, 261–262
 - early history, 30–31, 259–260
 - future research on, 276–277
 - histaminergic neuron activity, 318–319
 - mood disorders and, basic principles, 790
 - neurogenesis regulation, 831–833
 - neurotransmitter transporters (SERTs)
 - chronic substrate treatment, 726–727
 - clinical relevance, 720
 - glycosylation, 725–726
 - multiple transcription initiation sites, 725
 - promoter region polymorphisms, 725
 - serotonin degradation and reuptake, 261–262
 - stress-monoamine convergence in depression, 825–826
 - structure and function, 714
 - neurotrophins and, stress influence on, 797
 - receptors, 262–276
 - synthesis, 260–261
- Serotonin reuptake inhibitors (SRIs), hippocampal neurogenesis and depression and, 827
- Serum analysis, brain-derived neurotrophic factor in depressed patients, 801
- Severe myoclonic epilepsy of infancy (SMEI), voltage-gated sodium ion channels and, 642
- Short-term potentiation (STP), muscarinic acetylcholine receptor deficiency, 169
- SIB-1757, mGlu5 allosteric antagonists, 443
- SIB-1893, mGlu4 allosteric antagonists, 440–441
- Signaling molecules
- α_1 adrenergic receptor regulation, 203
 - α_2 -adrenergic receptor regulation, 206
 - β -adrenergic receptor activation, 210–211
 - calcium channels, 83–85
 - dopamine, 223–224
 - dopamine receptors, 230–232
 - GABA_A phosphorylation, 482
 - gaseous signaling
 - carbon monoxide, 756–757
 - future research, 757
 - nitric oxide
 - basic principles, 743–744
 - neurodegeneration and, 754
 - nitrosothiol detection, 747–748
 - nitrosylation mechanism, 745–747
 - cell survival, 752
 - extracellular matrix, 751–752
 - gene transport, 750–751
 - ion channels, 748–749

- Parkinson's disease and, 755–756
- protein-protein interactions, 749–750
- S nitrosylation physiology, 752–754
- vesicular transport, 751
- physiological role, 745
- research background, 743–744
- H₂ receptor, 312
- H₃ receptor, 314–315
- H₁ receptors, 309
- IP₃ and phosphoinositides, 81–83
- muscarinic acetylcholine receptor
 - modulation, 158–159
- voltage-gated calcium channels, 647
- Single-nucleotide polymorphisms (SNPs)
 - β -adrenergic receptor physiology, 212–213
 - dopamine, 234–236
 - plasma membrane neurotransmitter transporter regulation, 724–725
- Site-directed mutagenesis, neuropeptides, 679
- Skeletal muscle, voltage-gated sodium ion channels and, 641–642
- Skin function, muscarinic acetylcholine receptor deficiency, 177
- SLC1 family, plasma membrane glutamate transporters, 708–711
- SLC5 family, structure and function, 714–715
- SLC6 family of neurotransmitters, 711–714
- SLC32 transporter family, 718
- Small-conductance calcium-activated potassium channels (SK channels), structure and function, 631–632
- Small G proteins, structure and function, 63–64
- Smooth muscle function, muscarinic acetylcholine receptor deficiency, 173–174
- S nitrosylation
 - nitric oxide, protein mechanisms, 747
 - physiology of, 752–754
- Sodium channels
 - early research on, 25
 - plasma membrane glutamate transporters, 709–711
 - voltage-gated ion channels, 635–643
 - α subunits, 636–637
 - channelopathies, 641–642
 - genetics, 619–620
 - inactivation, 637–638
 - pharmacology, 642–643
 - physiological functions, 639–641
 - selectivity, 637
 - trafficking, 638–639
- Sodium/chloride-dependent neurotransmitter transporters, 711–714
 - dopamine transporters, 712–713
 - GABA transporters, 711–712
 - glycine transporters, 712
 - norepinephrine transporters, 713–714
 - serotonin transporters, 714
- Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)
 - plasma membrane transporter regulation, 728
 - vesicle-dependent release, 46–47
 - nitrosylation and, 751
- Somatostatin (ST), neuropeptides and, 671
- Somatotropin release-inhibiting factor (SRIF), neuropeptide hypothalamic control, 691–692
- Splice variants, dopamine, 234–236
- 1S,3R-ACPD, mGlu group I selective orthosteric agonists, 423
- Steroid hormones
 - GABA_A α_4 subunit pharmacology, 488–489
 - NMDA allosteric potentiators, 386
- Stoichiometry, plasma membrane glutamate transporters, 709–711
- Stress
 - histaminergic neuron activity, 320–322
 - pituitary hormone secretion, 325
 - hormones and, 679
 - neurogenesis regulation, 829–830
 - monoamine hypothesis of depression and, hippocampal neurogenesis and, 825–826
 - neuroendocrine response, 694–695
 - neurotrophic factors and, growth factors, 794–797
 - adrenal glucocorticoids, 796–797
 - adult neurogenesis and morphology, 797–800
 - hippocampus, 798
 - alterations in, 794–795
 - brain-derived neurotrophic factor, 794–796
 - cytokines, 797

- glial proliferation, 800
- serotonin receptors, 797
- vascular endothelial growth factor, 797
- Striatal complex
 - dopamine release, muscarinic acetylcholine receptor deficiency, 171–172
 - histaminergic neuron activity, Parkinson's disease, 328
- Stroke therapy
 - GABA transporters and, 722
 - mGlu II selective orthosteric agonists, 428–429
 - mGlu I selective orthosteric antagonists, 425
 - NMDA orthosteric antagonists, 375–377
- Structure-activity relationships
 - dopamine, 232–234
 - mGlu2 allosteric potentiators, 438–439
- Structure-affinity relationships, dopamine, 232–234
- Substance abuse
 - dopaminergic drugs for, 238–239
 - GABA_B receptor targeting, 593–594
 - 5-HT_{2C} receptors and, 272–273
 - muscarinic acetylcholine receptor deficiency, 173
 - serotonin transporters and, 720
- Substance P, neuropeptides and, 670–671
 - neurotransmitters, 676
- Subtype-selective ligands, GABA_B receptors, 592
- 6-Sulfatoxymelatonin (6-SMT), menopause therapy and, 849–850
- Surface trafficking, GABA_B receptors, 575–578
- Synapses
 - curare's effect on, 4–7
 - early research on, 13–14
 - Eccles' research on, 28–29
 - electric organ model of, 22
 - Feldberg's research on, 20–21
 - GABA_A receptor distribution on, 473–475
 - ion channels and, 25
 - Loewi's experiments on, 18–19
 - postwar research on, 24–25
 - serotonergic neurons, 264
- Synaptic release
 - vesicle-dependent release, 46–47
 - vesicle-independent release, 47–48
- voltage-gated sodium ion channels, 639–640
- Synaptic transmission
 - AMPA receptor allosteric potentiators, 394–395
- intercellular signaling
 - basic principles, 40–41
 - classical transmitters, 45–46
 - dopamine neurotransmitters, 54–56
 - endocannabinoids, purines, and gaseous transmitters, 46
 - glutamate neurotransmitters, 52–54
 - peptide transmitters and growth factors, 46
 - postsynaptic receptors, 48–50
 - presynaptic receptors, 50–51
 - synaptic release, 46–48
 - transmitter inactivation, 51–52
 - transmitter packaging, 45–46
 - transmitter synthesis, 41–45
 - amine transmitters, 42–44
 - endocannabinoids, 45
 - gaseous transmitters, 45
 - neuropeptides, neurotrophins, and growth factors, 44–45
 - vesicle-dependent release, 46–47
 - vesicle-independent release, 47–48
- intracellular signaling
 - basic principles, 59–60
 - calcium channel calmodulin mediator, 85–87
 - calcium channel signaling molecules, 83–85
 - cyclic nucleotide second messengers, 72–81
 - adenylyl cyclase, 72–77
 - cAMP targets, 75, 78
 - cGMP cellular targets, 80–81
 - guanylyl cyclase, 78–80
 - DAG activation of protein kinase C, 85
 - GPCR-G protein ion channel
 - modulation, 66–71
 - downstream signaling molecules, 69–70
 - G $\beta\gamma$ signaling, 67–69
 - protein-protein interactions, 70–71
 - G protein signal transducers, 60–66
 - cycle regulations, 64–65
 - disease and, 67
 - heterotrimeric protein structure, 60–63
 - RGS proteins, 65–66
 - small proteins, 63–64

- IP₃ and phosphoinositide signaling molecules, 81–83
- protein phosphorylation, 87–96
 - G-protein-coupled receptor kinases, 90–92
 - mitogen-activated protein kinase, 89–90
 - protein tyrosine kinase, 87–89
 - protein tyrosines phosphatases, 92–94
 - serine/threonine phosphatases, 94–96
- Synaptic vesicles, early research on, 27
- Synaptotagmin, vesicle-dependent release, 46–47
- Tachykinin, neuropeptide receptors, 689–690
- T-182C polymorphism, norepinephrine transporter genetics, 201
- + TC
 - actions of, 27
 - postwar applications of, 25
- Tele*-methylhistamine (*t*-MeHA),
 - histaminergic neuron activity, 319–322
- Tetraethylammonium (TEA), voltage-gated potassium channels, K_V subunits, structure and function, 630
- Tetrodotoxin (TTX), voltage-gated sodium ion channels and, 642–643
- Thalamic relay neurons, histamine neuron physiology, arousal mechanisms, 322–324
- Thalamic reticular nucleus, GABA_A α₃ subunit pharmacology, 488
- Therapeutic targeting, GABA_B receptors, 592–593
- Thyroid hormones, GABA_A receptor modulation, 520
- Thyroid-stimulating hormone (TSH)
 - depression and levels of
 - in menopause, 850
 - during menstrual cycle, 845
 - postpartum depression, 848
 - in pregnancy, 847
 - neuropeptides and, 676–677
- Time-sensitive responses, to neuropeptides, 693–694
- Tissue culture studies
 - brain-derived neurotrophic factor in depressed patients, 800–801
 - neuropeptides, 677
- Tissue-specific processing, neuropeptides, 687, 693–694
- Torpedo* electric organ
 - GABA_A receptor structure and, 475
 - nicotinic acetylcholine receptor history, 108–110
 - synapse research and, 22–23
- Torpedo* electric organ model
 - nicotinic acetylcholine receptor, 27–28
 - of synapse activity, 22
- Torsade de pointes, KCNQ channels and, 632–633
- Tourette's syndrome, dopaminergic drugs, 237
- Trafficking mechanisms
 - GABA_A receptors, 478–480
 - GABA_B receptors, 575–578
 - voltage-gated sodium ion channels, 638–639
- TRAM-34 analog,
 - intermediate-conductance calcium-activated potassium channels, 631
- Trans*-aminocyclobutanedicarboxylate (ABCD), NMDA orthosteric agonists, 373
- Transcriptional regulation
 - α₁ adrenergic receptors, 202–203
 - β-adrenergic receptor physiology, 212–213
 - lithium mechanism in bipolar disorder patients, 867–869
 - nitrosylation and, 750–751
 - plasma membrane transporters, 725
- Transforming growth factor-β (TGF-β),
 - intracellular signaling, 793–794
- Transgenic animals, neuropeptide genetic manipulations in, 678
- Trans-Golgi network (TGN), GABA_B receptor trafficking and heteromization, 577–578
- Transient receptor potential (TRP) channels
 - cyclic nucleotide second messenger signaling, adenylyl cyclase, 74–75
 - structure and function, 649–650
- Translocation, plasma membrane glutamate transporters, 710–711
- Transmembrane diffusion, blood-brain barrier neuropeptides and, 692–693
- Transmembrane domains (TMDs)
 - AMPA receptors, 370–371
 - dopamine, 233–234

- GABA_A receptors
 - allosteric sites in, 516–518
 - allosteric structural determinants, 508–509
 - channel gating binding, 495–497
 - desensitization and deactivation, 499
 - subunit gene splicing, 469–470
- GABA transporters, 467–468
- mGlu5 allosteric antagonists, 443–444
- mGlu1 allosteric potentiators, 436
- muscarinic acetylcholine receptor
 - structure, 149–151
- plasma membrane glutamate transporters, 709–711
- sodium voltage-gated ion channels, 636
- voltage-gated calcium channels, γ subunits, 646
- Transmembrane segment (TMS) topology, voltage-gated ion channels, 619–620
- Transmethylation, histamine inactivation, 306–307
- Transmitter inactivation, intercellular signaling, 51–52
- Transmitter packaging, classical transmitters, 45–46
- Transmitter synthesis, intercellular signaling and, 41–45
 - amine transmitters, 42–44
 - endocannabinoids, 45
 - gaseous transmitters, 45
 - neuropeptides, neurotrophins, and growth factors, 44–45
- Tremor induction, muscarinic acetylcholine receptor deficiency, 170–171
- Tricarboxylic acid (TCA) cycle, plasma membrane glutamate transporters, 711
- Trichloroethanol, GABA_A receptor modulation, 516
- Tricyclic antidepressants, early research on, 30–31
- Tryptophan hydroxylase (TPH), serotonin synthesis, 260–261
- Tuberomammillary nucleus
 - histamine neuron physiology
 - arousal mechanisms, 323–324
 - cognitive function, 324
 - histaminergic neuron activity, 319–322
 - histaminergic neurons, 301–303
- Tubocurarine, small-conductance calcium-activated potassium channels, 632
- Tumor cell growth and migration
 - GABA_B receptor targeting, 596
 - KCNH potassium channels, 634
- Two-P (KCNK) channels, structure and function, 634–635
- Tyrosine kinase receptors
 - chemical release, 48–50
 - GABA_A phosphorylation, 482
 - glutamate transporter regulation, 727–728
 - neuropeptide receptors, 691
- Uncoupling reactions, muscarinic acetylcholine receptors, 162
- Upregulation, neuropeptide receptors, 689
- val* alleles, BDNF in mood disorders, 803
- Variable number of tandem repeats (VNTRs)
 - plasma membrane transporter regulation, 725
 - serotonin transporters and, 720
- Vascular endothelial growth factor (VEGF)
 - antidepressant influences on, 805–806
 - adult neurogenesis and, 807–808
 - depression models and, 809
 - intracellular signaling, 792–793
 - stress influence on, 797
 - neurogenesis and, 799–800
- Vascular tone, α_1 adrenergic receptor physiology, 203–204
- Vasoactive intestinal polypeptide (VIP), neuropeptides and, 670–671
 - modulation actions, 677
- Vasopressin, neuropeptides and gene duplication and, 682–684
 - modulation, 677
- Vasopressinergic neurons, H₁ receptor signaling, 309
- Ventrolateral preoptic nucleus (VLPO)
 - histaminergic neuron activity, 319–322
 - histaminergic neurons, 305
- Vesicle-dependent synaptic release, 46–47
- Vesicle-independent synaptic release, 47–48
- Vesicular GABA transporters (VGATs)
 - function of, 466
 - GABA affinity, 467–468
- Vesicular glutamate transporters (VGLUT)
 - glutamate synthesis and storage, 366
 - neurotransmitter functions, 716, 723
- Vesicular inhibitory amino acid transporters, structure and function, 718

- Vesicular monoamine transporter 2 (VMAT 2)
 - chronic substrate treatment, 726–727
 - histamine metabolism, 306
 - neurotransmitter functions, 716–717, 723
- Vesicular neurotransmitter transporters, 715–718
 - acetylcholine and monoamine transporters, 716–718
 - clinical relevance, 723
 - glutamate transporters, 716
 - inhibitor amino acid transporters, 718
 - nitrosylation and, 751
- Vogel conflict model, mGlu5 allosteric antagonists, 444
- Voltage activation, potassium channels, 625–626
- Voltage-gated calcium channels (VGCCs)
 - downstream signaling, 69–70
 - signal modulation, 68–69
- Voltage-gated ion channels
 - calcium channels, 643–650
 - α subunits, 644–645
 - $\alpha\delta$ subunits, 646
 - auxiliary subunit modulators, 650
 - β subunits, 645–646
 - Ca_v1 family, 647–648
 - Ca_v2 family, 648–649
 - Ca_v3 family, 649–650
 - γ subunits, 646
 - general blockers, 647
 - miscellaneous channels, 650–651
 - cloning and evolutionary relationships, 619–620
 - nomenclature, 621
 - potassium channels, 621–635
 - calcium activation, 630–632
 - fast inactivation, 627
 - gating, 626–627
 - interacting proteins, 627–628
 - inwardly-rectifying and two-P channels, 634–635
 - KCNH channels, 633–634
 - KCNQ family, 632–633
 - K_v subunits, 628–630
 - physiology, disease and pharmacology, 628–634
 - selectivity, 623–625
 - voltage activation, 625–626
- research background, 618–619
- sodium channels, 635–643
 - α subunits, 636–637
 - channelopathies, 641–642
 - inactivation, 637–638
 - pharmacology, 642–643
 - physiological functions, 639–641
 - selectivity, 637
 - trafficking, 638–639
- Weibel Palade bodies, nitrosylation and, 751
- Willardiines
 - AMPA receptor orthosteric agonists, 376–377
 - kainate receptor orthosteric agonists, 380–381
 - kainate receptor orthosteric antagonists, 384–385
- Zaleplon, GABA_A subunit pharmacology, allosteric ligand modulation, 504–506
- Ziconotide, voltage-gated calcium ion channels, Ca_v2 family, 649
- Zolpidem
 - GABA_A subunit pharmacology
 - allosteric ligand modulation, 504–506
 - benzodiazepine binding sites, 510–511
 - GABA_A subunit selectivity, 484

PREFACE

Neuropharmacology is the study of drugs that affect the nervous system. This includes not only the identification of neuronal drug targets but also the study of basic mechanisms of neural function that may be amenable to pharmacological manipulation. Indeed, neuropharmacological drugs are commonly used as valuable tools to discover how nerve cells function and communicate in addition to therapeutic agents for the treatment of a wide variety of neuropsychiatric disorders. In fact, drugs that are used to treat disorders of the brain and nervous system represent one of the largest groups of approved therapeutic agents. Clearly the demand for drugs to treat disorders of the nervous system will only grow in the face of an aging population. Not surprisingly, almost all major pharmaceutical corporations and many biotechnology companies have extensive drug discovery programs in neuroscience and neuropharmacology. The recent pace of research and discovery in neuropharmacology and associated therapeutics has been quite rapid, as is true for most areas of biomedical research. Given this as well as the extremely broad nature of the field, we felt that it would be timely and important to develop a comprehensive handbook of neuropharmacology that would include state-of-art reviews covering both basic principles and novel approaches for clinical therapeutics.

Our approach for the organization of this handbook was primarily translational (bench to bedside) in nature. The three book volumes consist of 10 clinical sections, each consisting of 4–7 chapters devoted to various neuropsychiatric disorders, including mood, anxiety, and stress disorders, psychosis, pain, neurodegeneration, and many others. In most cases, these sections have introductory chapters providing background information and/or basic principles prior to presenting chapters covering state-of-the-art therapeutics. Volume I also contains a large introductory section consisting of 17 chapters on basic neuropharmacological subjects and principles. These include chapters on the history of neuropharmacology as well as intercellular and intracellular signaling followed by chapters covering all of the major neurotransmitter systems and other important signaling molecules, such as ion channels and transporters. Our objective for this project was to create a high-level reference work that will be useful to all practitioners of neuropharmacology ranging from graduate students, academicians, and clinicians to industrial scientists working in drug discovery. These volumes will be part of the John Wiley & Sons major reference work program and will be published online as well as in print. The online version of this handbook is expected to undergo frequent updates and additions in order to maintain its cutting-edge status.

The editors would like to thank all of the chapter contributors for their hard work and commitment to this project. We would also like to thank our managing editor, Jonathan Rose, at John Wiley & Sons for all of the valuable assistance that he has provided.

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Contents

Preface	xi
Contributors	xiii

VOLUME 1

PART I BASIC NEUROPHARMACOLOGY	1
Chapter 1 Soup or Sparks: The History of Drugs and Synapses	3
<i>William Van der Kloot</i>	
Chapter 2 Synaptic Transmission: Intercellular Signaling	39
<i>J. David Jentsch and Robert H. Roth</i>	
Chapter 3 Synaptic Transmission: Intracellular Signaling	59
<i>R. Benjamin Free, Lisa A. Hazelwood, Yoon Namkung Michele L. Rankin, Elizabeth B. Rex, and David R. Sibley</i>	
Chapter 4 Neuronal Nicotinic Receptors: One Hundred Years of Progress	107
<i>Kenneth J. Kellar and Yingxian Xiao</i>	
Chapter 5 Muscarinic Acetylcholine Receptors	147
<i>Jürgen Wess</i>	
Chapter 6 Norepinephrine/Epinephrine	193
<i>Megan E. Kozisek and David B. Bylund</i>	
Chapter 7 Dopaminergic Neurotransmission	221
<i>John A. Schetz and David R. Sibley</i>	
Chapter 8 Serotonin Systems	257
<i>John A. Gray and Bryan L. Roth</i>	
Chapter 9 Neuropharmacology of Histamine in Brain	299
<i>Raphaël Faucard and Jean-Charles Schwartz</i>	

Chapter 10	Ionotropic Glutamate Receptors	365
	<i>David Bleakman, Andrew Alt, David Lodge, Daniel T. Monaghan, David E. Jane, and Eric S. Nisenbaum</i>	
Chapter 11	Metabotropic Glutamate Receptors	421
	<i>James A. Monn, Michael P. Johnson, and Darryle D. Schoepp</i>	
Chapter 12	Pharmacology of the GABA_A Receptor	465
	<i>Dmytro Berezhnoy, Maria C. Gravielle, and David H. Farb</i>	
Chapter 13	Metabotropic GABA Receptors	569
	<i>Martin Gassmann and Bernhard Bettler</i>	
Chapter 14	Voltage-Gated Ion Channels	617
	<i>Alex Fay, Patrick C. G. Haddick, and Lily Yeh Jan</i>	
Chapter 15	Neuropeptides	669
	<i>Fleur L. Strand</i>	
Chapter 16	Neurotransmitter Transporters	705
	<i>Jia Hu, Katherine Leitzell, Dan Wang, and Michael W. Quick</i>	
Chapter 17	Gaseous Signaling: Nitric Oxide and Carbon Monoxide as Messenger Molecules	743
	<i>Kenny K. K. Chung, Valina L. Dawson, and Ted M. Dawson</i>	
PART II	MOOD DISORDERS	763
Chapter 18	Neurobiology and Treatment of Depression	765
	<i>Alexander Neumeister, Dennis S. Charney, Gerard Sanacora, and John H. Krystal</i>	
Chapter 19	Neurotrophic Factors in Etiology and Treatment of Mood Disorders	789
	<i>Ronald S. Duman</i>	
Chapter 20	Antidepressant Treatment and Hippocampal Neurogenesis: Monoamine and Stress Hypotheses of Depression Converge	821
	<i>Alex Dranovsky and René Hen</i>	
Chapter 21	Neuroendocrine Abnormalities in Women with Depression Linked to the Reproductive Cycle	843
	<i>Barbara L. Parry, Charles J. Meliska, L. Fernando Martinez, Eva L. Maurer, Ana M. Lopez, and Diane L. Sorenson</i>	
Chapter 22	Neurobiology and Pharmacotherapy of Bipolar Disorder	859
	<i>R. H. Belmaker, G. Agam, and R. H. Lenox</i>	
Index		877
Cumulative Index		915

VOLUME 2

PART I	ANXIETY AND STRESS DISORDERS	1
Chapter 1	Neurobiology of Anxiety	3
	<i>Miklos Toth and Bojana Zupan</i>	
Chapter 2	Pharmacotherapy of Anxiety	59
	<i>Jon R. Nash and David J. Nutt</i>	
Chapter 3	Benzodiazepines	93
	<i>Hartmut Lüddens and Esa R. Korpi</i>	
Chapter 4	Neuroactive Steroids in Anxiety and Stress	133
	<i>Deborah A. Finn and Robert H. Purdy</i>	
Chapter 5	Emerging Anxiolytics: Corticotropin-Releasing Factor Receptor Antagonists	177
	<i>Dimitri E. Grigoriadis and Samuel R. J. Hoare</i>	
Chapter 6	Neurobiology and Pharmacotherapy of Obsessive-Compulsive Disorder	215
	<i>Judith L. Rapoport and Gale Inoff-Germain</i>	
PART II	SCHIZOPHRENIA AND PSYCHOSIS	249
Chapter 7	Phenomenology and Clinical Science of Schizophrenia	251
	<i>Subroto Ghose and Carol Tamminga</i>	
Chapter 8	Dopamine and Glutamate Hypotheses of Schizophrenia	283
	<i>Bitá Moghaddam and Houman Homayoun</i>	
Chapter 9	Molecular Genetics of Schizophrenia	321
	<i>Liam Carroll, Michael C. O'Donovan, and Michael J. Owen</i>	
Chapter 10	Postmortem Brain Studies: Focus on Susceptibility Genes in Schizophrenia	343
	<i>Shiny V. Mathew, Shruti N. Mitkus, Barbara K. Lipska, Thomas M. Hyde, and Joel E. Kleinman</i>	
Chapter 11	Pharmacotherapy of Schizophrenia	369
	<i>Zafar Sharif, Seiya Miyamoto, and Jeffrey A. Lieberman</i>	
Chapter 12	Atypical Antipsychotic Drugs: Mechanism of Action	411
	<i>Herbert Y. Meltzer</i>	

PART III	SUBSTANCE ABUSE AND ADDICTIVE DISORDERS	449
Chapter 13	Introduction to Addictive Disorders: Implications for Pharmacotherapies	451
	<i>Mary Jeanne Kreek</i>	
Chapter 14	Dopaminergic and GABAergic Regulation of Alcohol-Motivated Behaviors: Novel Neuroanatomical Substrates	465
	<i>Harry L. June and William J. A. Eiler II</i>	
Chapter 15	Nicotine	535
	<i>August R. Buchhalter, Reginald V. Fant, and Jack E. Henningfield</i>	
Chapter 16	Psychostimulants	567
	<i>Leonard L. Howell and Heather L. Kimmel</i>	
Chapter 17	MDMA and Other “Club Drugs”	613
	<i>M. Isabel Colado, Esther O’Shea, and A. Richard Green</i>	
Chapter 18	Marijuana: Pharmacology and Interaction with the Endocannabinoid System	659
	<i>Jenny L. Wiley and Billy R. Martin</i>	
Chapter 19	Opiates and Addiction	691
	<i>Frank J. Vocci</i>	
PART IV	PAIN	707
Chapter 20	Neuronal Pathways for Pain Processing	709
	<i>Gavril W. Pasternak and Yahong Zhang</i>	
Chapter 21	Vanilloid Receptor Pathways	727
	<i>Makoto Tominaga</i>	
Chapter 22	Opioid Receptors	745
	<i>Gavril W. Pasternak</i>	
Chapter 23	Advent of A New Generation of Antimigraine Medications	757
	<i>Ana Recober and Andrew F. Russo</i>	
	Index	779
	Cumulative Index	817
VOLUME 3		
PART I	SLEEP AND AROUSAL	1
Chapter 1	Function and Pharmacology of Circadian Clocks	3
	<i>Gabriella B. Lundkvist and Gene D. Block</i>	

Chapter 2	Melatonin Receptors in Central Nervous System	37
	<i>Margarita L. Dubocovich</i>	
Chapter 3	Narcolepsy: Neuropharmacological Aspects	79
	<i>Seiji Nishino</i>	
Chapter 4	Hypocretin/Orexin System	125
	<i>J. Gregor Sutcliffe and Luis de Lecea</i>	
Chapter 5	Prokineticins: New Pair of Regulatory Peptides	163
	<i>Michelle Y. Cheng and Qun-Yong Zhou</i>	
Chapter 6	Sedatives and Hypnotics	177
	<i>Keith A. Wafford and Paul J. Whiting</i>	
PART II	DEVELOPMENT AND DEVELOPMENTAL DISORDERS	201
Chapter 7	Regulation of Adult Neurogenesis	203
	<i>Heather A. Cameron</i>	
Chapter 8	Neurotrophic Factors	221
	<i>Franz F. Hefti and Patricia A. Walicke</i>	
Chapter 9	Neurotrophins and Their Receptors	237
	<i>Mark Bothwell</i>	
Chapter 10	Tourette's Syndrome and Pharmacotherapy	263
	<i>Pieter Joost van Watum and James F. Leckman</i>	
Chapter 11	Neuropharmacology of Attention-Deficit/Hyperactivity Disorder	291
	<i>Paul E. A. Glaser, F. Xavier Castellanos, and Daniel S. Margulies</i>	
Chapter 12	Psychopharmacology of Autism Spectrum Disorders	319
	<i>Adriana Di Martino, Steven G. Dickstein, Alessandro Zuddas, and F. Xavier Castellanos</i>	
PART III	NEURODEGENERATIVE AND SEIZURE DISORDERS	345
Chapter 13	Stroke: Mechanisms of Excitotoxicity and Approaches for Therapy	347
	<i>Michael J. O'Neill, David Lodge, and James McCulloch</i>	
Chapter 14	Epilepsy: Mechanisms of Drug Action and Clinical Treatment	403
	<i>William H. Theodore and Michael A. Rogawski</i>	
Chapter 15	Pharmacotherapy for Traumatic Brain Injury	443
	<i>Donald G. Stein and Stuart W. Hoffman</i>	
Chapter 16	Dementia and Pharmacotherapy: Memory Drugs	461
	<i>Jerry J. Buccafusco</i>	

Chapter 17	Pharmacotherapy and Treatment of Parkinson's Disease	479
	<i>Wing Lok Au and Donald B. Calne</i>	
Chapter 18	Parkinson's Disease: Genetics and Pathogenesis	523
	<i>Claudia M. Testa</i>	
Chapter 19	Invertebrates as Powerful Genetic Models for Human Neurodegenerative Diseases	567
	<i>Richard Nass and Charles D. Nichols</i>	
PART IV	NEUROIMMUNOLOGY	589
Chapter 20	Myelin Lipids and Proteins: Structure, Function, and Roles in Neurological Disorders	591
	<i>Richard H. Quarles</i>	
Chapter 21	Pharmacology of Inflammation	621
	<i>Carmen Espejo and Roland Martin</i>	
Chapter 22	Pharmacological Treatment of Multiple Sclerosis	671
	<i>B. Mark Keegan</i>	
Chapter 23	Novel Therapies for Multiple Sclerosis	683
	<i>Martin S. Weber and Scott S. Zamvil</i>	
Chapter 24	Neuropharmacology of HIV/AIDS	693
	<i>Sidney A. Houff and Eugene O. Major</i>	
PART V	EATING AND METABOLIC DISORDERS	731
Chapter 25	Leptin: A Metabolic Perspective	733
	<i>Dawn M. Penn, Cherie R. Rooks, and Ruth B. S. Harris</i>	
Chapter 26	Ghrelin: Structural and Functional Properties	765
	<i>Birgitte Holst, Kristoffer Egerod, and Thue W. Schwartz</i>	
Chapter 27	Mechanisms Controlling Adipose Tissue Metabolism by the Sympathetic Nervous System: Anatomical and Molecular Aspects	785
	<i>Sheila Collins, Renato H. Migliorini, and Timothy J. Bartness</i>	
Chapter 28	Antiobesity Pharmacotherapy: Current Treatment Options and Future Perspectives	815
	<i>Yuguang Shi</i>	
Index		845
Cumulative Index		881

1

NEUROBIOLOGY OF ANXIETY

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1.1	Introduction	4
1.2	Psychological Traits and their Genetic Basis	4
1.3	Extrapolation of Psychological Trait of Neuroticism to Mouse Behavior	6
1.3.1	Emotionality as Measure of Avoidance, Behavioral Inhibition/ Activation, and Autonomic Arousal in Animals	6
1.3.2	Quantifying Emotionality in Animals	6
1.4	Anxiety: Continuous Expression of Normal Human Personality Traits	9
1.4.1	Anxiety Disorders	9
1.4.2	Anxiety-Like Behavior in Animals	10
1.5	Fear/Anxiety Circuits	13
1.5.1	Brain Regions Related to Anxiety Disorders	13
1.5.2	Brain Regions Related to Emotionality/Anxiety-Like Behavior in Animals	14
1.6	Neurotransmitter Systems and Neuronal Messengers Implicated in Anxiety and Anxiety-like Behavior	15
1.7	Genetic Susceptibility to Anxiety Disorders	18
1.8	Genetic Base of Anxiety-like Behavior in Mice	19
1.8.1	QTL Studies	19
1.8.2	Anxiety-Like Behavior in Genetically Altered Mice	19
1.9	Knockout Mice with Disturbances in Neuronal Messengers Exhibiting Alterations in Anxiety-like Behavior	24
1.10	Knockout Mice with Deficits in Neurotransmitter Receptors and Other Cytoplasmic Membrane-associated Proteins Exhibiting Anxiety-like Behavior	26
1.11	Intracellular Regulators Associated with Anxiety-like Phenotype	30
1.11.1	Modeling Complex Genetics of Anxiety in Mice: Oligogenic Anxiety-Like Conditions in Mice	32
1.12	Effects of Early-Life Environment on Anxiety	33
1.12.1	Early-Life Experience on Expression of Anxiety in Later Life	33
1.12.2	Interaction of Environment with Genes in Establishing Level of Anxiety	34
1.13	Conclusions: Neurobiology of Anxiety Disorders	35
	References	37

1.1 INTRODUCTION

“Anxiety” is the subjective feeling of heightened tension and diffused uneasiness. It is a normal reaction to threatening situations and serves a physiological protective function in eliciting avoidance behaviors. The majority of individuals respond to anxiety-evoking environment appropriately but with some individual differences. The range of appropriate responses to threatening situations can best be described by individual differences in personality traits, in particular in emotional (in)stability/neuroticism [1–3]. Both genetic and environmental factors contribute to emotional (in)stability and to personality traits in general [4]. Approximately 5–10% of individuals display an exaggerated response to real or perceived threat or interpret ambiguous situations as threatening and can be classified as suffering from anxiety disorders [5]. It may be conceptualized that these individuals lie outside of the normal range of individual differences in emotional (in)stability [6]. Indeed, emotional instability and anxiety share common genetic factors.

Although a genetic contribution to emotional instability (neuroticism) and anxiety has long been known, it is only recently that multipoint linkage analysis identified chromosomal regions that may harbor candidate genes [7, 8]. Also, genetic polymorphisms in the serotonin (5-HT) transporter (5-HTT), 5-HT_{1A} receptor, and brain-derived neurotrophic factor (BDNF) have recently been associated with neuroticism and anxiety conditions [9–11]. The slow pace of discovering susceptibility genes in human is largely due to the complex genetics of personality traits and common disorders; thus, individual genes have a relatively small contribution to traits/diseases. The effect of the environment on the expression of anxiety disorders also complicates the elucidation of the underlying pathogenic processes.

Animal models have long been used in the research of anxiety. Quantitative trait locus (QTL) analysis suggests that the genetic basis of “emotionality”/fear reaction in mice can be defined as the variance of a set of few behavioral measures [12–15], such as avoidance of novel environment (abbreviated in this review as Av), behavioral inhibition/activity in highly or moderately threatening situations (Ac) and autonomic arousal (Aa). These dimensions of rodent behavior are reminiscent of the characteristics of emotional instability/neuroticism in humans. In the last decade, a large number of induced mutations have been generated by homologous recombination in the mouse, and some of these strains show a significant deviation from their parental strain in measures of fear response. The abnormal fear response of these mice can be conceptualized as anxiety-like, similar to anxiety disorders in humans. By analyzing a large number of these strains and by classifying them according to the three fundamental dimensions of noncognitive behavior proposed above (AvAcAa), it is possible to implicate multiple neurobiological processes in anxiety-like behavior. Furthermore, these genetic models allow the study of the combined effect of two or more genes as well as the interaction of genes and environment in the expression of anxiety-like behavior in mice. These results may be extrapolated to humans and they eventually could help to better understand the polygenic and multifactorial nature of human anxiety disorders.

1.2 PSYCHOLOGICAL TRAITS AND THEIR GENETIC BASIS

Since anxiety is the continuous expression of normal human personality traits, it is important to briefly summarize a few of the leading personality theories. Personality

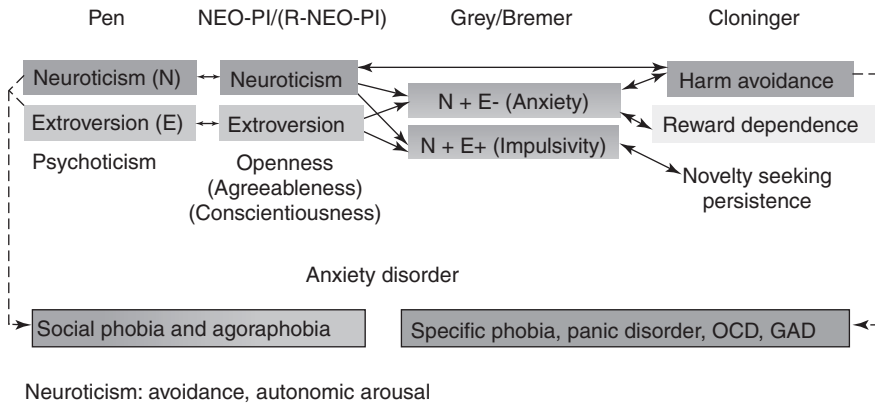


Figure 1.1 Psychological traits and models. According to various models, personality consists of three to five fundamental traits. Arrows indicate corresponding traits in the various models. Traits are highly variable in individuals, but most individuals fall in between the extremes of a trait. Behavior outside of this range can be considered abnormal. For example, higher than normal level of neuroticism (in the PEN model) or harm avoidance (in the Cloninger model) is characteristic for anxiety disorders. (See color insert.)

traits are underlying characteristics of an individual that can explain the major dimensions of human behavior. Traits are dimensions representing a continuum of characters and most people fall in between the extremes. Personality traits have a wide individual variation, but they are relatively stable in individuals over time [16]. Cognitive/intellectual and noncognitive/affective/psychological traits are two fundamental domains of personality. Although the separation of the cognitive and noncognitive domains of personality may be practical, these variables interact and influence each other. Among the psychological traits two, extroversion versus introversion (E) and emotional stability versus instability or neuroticism (N), are probably the most important. An additional dimension is psychoticism (P) in the PEN model [1, 2] (Fig. 1.1). Autonomic arousal is an integral part of neuroticism and it is characterized by increased heart rate and blood pressure, cold hands, sweating, and muscular tension. A similar system based on the broad traits of neuroticism, extroversion, and openness is the NEO personality inventory (NEO-PI) [3]. Other models hypothesize the existence of more than three fundamental traits. The Big 5 (B5) model has three other dimensions in addition to emotional stability and extroversion [17–19]. The revised (R) NEO-PI also has five factors, and besides neuroticism, extroversion, and openness, consists of the factors of agreeableness and conscientiousness [20] (Fig. 1.1). NEO-PI-R is a self-report inventory with a high retest reliability, item validity, longitudinal stability, consistent correlations between self and observer ratings, and robust factor structure that has been validated in a variety of populations and cultures [3]. Gray [21] has modified Eysenck's PEN model by rotating the dimensions of neuroticism and extroversion by 45°, resulting in two new dimensions: anxiety (N +, E –) and impulsivity (N +, E +). Gray's work, however, has been done mostly on animals. Still another personality assessment is Cloninger's biosocial model, which conceptualizes temperament as consisting of the four genetically and biochemically distinct traits of harm avoidance, reward

dependence, novelty seeking, and persistence [22] (Fig. 1.1). Harm avoidance is correlated with NEO-PI-R neuroticism. Reward dependence is related to the anxiety/neuroticism/extroversion traits of other classifications (Fig. 1.1). Novelty seeking is also related to these traits and is similar to impulsivity in the Gray hypothesis. Each of these broad dimensions of personality is comprised of a number of smaller traits which are narrower in scope.

Using the techniques of quantitative behavioral genetics, it became clear that roughly 40–60% of the variation in most personality traits has a genetic base. Broad personality traits are under polygenic influence [4, 23]. Recently, genomewide linkage studies have been performed by using the EPQ (Eysenck personality questionnaire) [1, 2] to identify chromosomal regions associated with neuroticism. A two-point and multipoint nonparametric regression identified 1q, 4q, 7p, 8p, 11q, 12q, and 13q [7], while another similar study using multipoint, nonparametric allele sharing and regression identified 1q, 3centr, 6q, 11q, and 12p [8], confirming some of the linkages in the previous study.

1.3 EXTRAPOLATION OF PSYCHOLOGICAL TRAIT OF NEUROTICISM TO MOUSE BEHAVIOR

1.3.1 Emotionality as Measure of Avoidance, Behavioral Inhibition/Activation, and Autonomic Arousal in Animals

Behavioral studies with various rodent strains indicate that a set of a few behavioral measures can describe “emotionality,” a behavior similar to the psychological traits of emotional instability/neuroticism in humans [12–15]. To be able to analyze and compare a large number of animal studies, we have selected throughout this review three commonly used measures of emotionality: avoidance of novel environment, activity/behavioral inhibition in highly or moderately threatening situations, and autonomic arousal (Fig. 1.2). Here we refer to this triad of behavioral measures as AvAcAa (avoidance, activity, and arousal).

1.3.2 Quantifying Emotionality in Animals

Attempts to measure emotionality and stress response in rodents have yielded a large number of tests [24–28]. Ten years ago it was estimated that there were over 30 such tests in use [29], and modifications of earlier tests have likely increased this number since then. Initially, the development of these tests was facilitated by the need of preclinical identification and characterization of anxiolytics. Indeed, these tests are often referred to as anxiety tests, anxiety-related tests, or animal models of anxiety, even if most of them actually measure the normal reaction of animals to novelty and stress.

The animal models measure either unconditioned or conditioned fear/anxiety-like behaviors. Another classification is based on more specific behaviors such as social and defensive behavior. Table 1.1 provides a short list of the more commonly used tests while more thorough reviews of the assays can be found elsewhere [28–32]. Unconditioned exploration tests measure the natural conflict experienced by animals to either explore a novel environment for food, water, or social reward or avoid it due

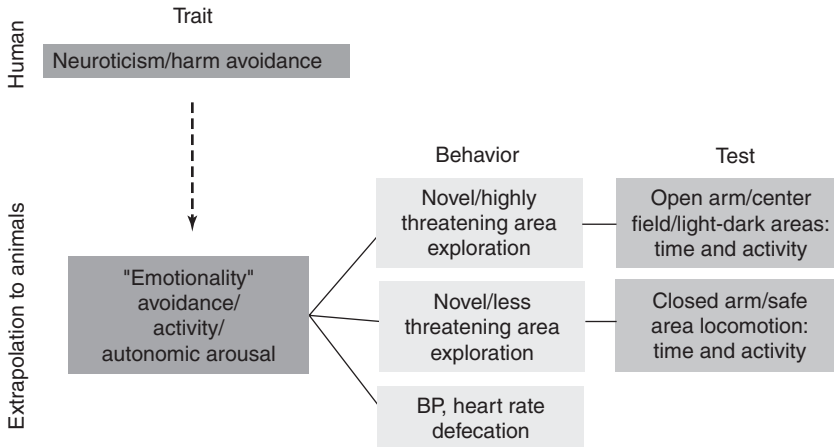


Figure 1.2 Measures of “emotionality” in rodents. The human trait of neuroticism is extrapolated to the measures of emotionality in rodents: avoidance, activity, and autonomic arousal (AvAcAa). AvAcAa can be quantified in well-established behavioral models. Exploration of a low and a moderate to highly threatening environment provides measures of avoidance and activity, while physiological functions provide measurements of autonomic arousal. (See color insert.)

TABLE 1.1 Commonly Used Animal Tests of Anxiety

Conditioned tests	<i>Punishment-Induced:</i> Geller–Seifter conflict, Vogel punished drinking	<i>Fear:</i> fear-potentiated startle, contextual/cued fear conditioning, passive/active avoidance	
Unconditioned tests	<i>Exploration:</i> elevated-plus and zero mazes, open field, light–dark box	<i>Social Interactions:</i> maternal separation, social competition	<i>Other:</i> acoustic startle, hyponeophagia, defense test batteries, shock-probe burying

to potential unknown dangers. Measurements of avoidant behaviors, such as decreased exploration of a particular region of the testing apparatus, compared to overall locomotor activity provide a quantifiable measure to assess the level of conflict in such novel environments. In a laboratory setting, animals are introduced into a novel and more or less fearful environment and their avoidance, behavioral inhibition/activity, and autonomic responses are measured. For example, the elevated-plus maze (EPM) [33] consists of a cross with opposing pairs of arms which are either open or enclosed and is elevated above the ground. The normal rodent behavior is to prefer the enclosed compartment of the maze, which is less aversive. During normal exploratory activity, however, the animal will enter the open arms. These entries into and the time spent in the open arms are counted and used to assess the level of avoidance, although additional, more complex behaviors can also be

recorded [33]. The elevated-zero maze (EZM) is a modification of the EPM where the four arms have been replaced by a circular track separated into four quadrants of alternating open and enclosed regions. Avoidance can also be assessed in the center of a brightly lit open field [34]. Animals tend to stay and move around the periphery of the field, since the open area and bright illumination are aversive. The avoidance measured in an open field is assessed by the number of entries into and the time spent in the center of the open field or the path length in this area. An adaptation of this test is the light–dark crossing task [35], consisting of a two-compartment box in which one area is dark and the other is brightly lit. This test uses the animals' natural tendency to prefer the dark and to avoid the brightly lit area. In this case, the number of crosses into and the time spent in the light compartment reflect the level of avoidance. Most studies compare the open arm/light compartment/center field activity/time as a percent of total activity/time. Also, exploration in the less aversive areas of the test apparatus (closed arm/dark compartment, periphery of the open field) is often regarded as an assessment of general activity levels, and it has been proposed that avoidance in the more stressful areas cannot be interpreted unless locomotor activity in low-stress areas is normal [36]. However, out-of-cage test environments are stressful even if they are moderately threatening. This notion is supported by the finding that a QTL has been linked to both the suppression of general locomotor activity and high-stress-area avoidance in a study involving a large number of mice [15]. Therefore, a number of laboratories, including ours, prefer to score overall locomotor activity separately (e.g., in activity boxes) as one measure of emotionality [37, 38]. The EPM, open field, and light–dark box tests are viewed as straightforward and relatively simple tests to conduct and as such are frequently used. However, more complex methods which highlight different aspects of avoidant behavior are available but are less commonly used. For example, in the social interaction test, behavior such as sniffing, grooming, mounting, and contact are monitored and used to infer changes in emotionality [39].

Conditioned conflict tests assess punishment-induced avoidance of a conditioned behavior. The Geller–Seifter test [40] is based on the conflict between completing an appetitive conditioned response that is unexpectedly paired with an unpleasant stimulus, such as the delivery of a mild electric shock. The Vogel punished drinking test [41] is similar to the Geller–Seifter test but does not require an extensive training period for the conditioning of the measured response. In the Vogel punished drinking test, the subject is water deprived for 12–24 h and then placed into a testing apparatus containing a water bottle with a spigot from which the animal can drink. Thirsty subjects learn quickly that water is available from the spigot and will readily drink from it when repeatedly placed into the testing apparatus. During the test session, however, the water spigot is connected to an electrical source that provides a mild electric shock upon contact with the spigot, placing the animal in conflict of choosing the appetitive reward or avoiding it. The level of avoidance reflects the emotionality of the subject.

Emotionality can also be measured in conditioned fear paradigms [42] such as fear-potentiated startle and contextual fear conditioning. These tests involve the element of emotional learning, as a neutral stimulus such as sound or light is paired with an electric foot shock. After a few trials, the previously neutral stimulus becomes aversive when presented alone. Mobility and freezing time can be used as indices of behavioral inhibition. A similar test is passive/active avoidance, a

one-pairing fear-induced avoidance assay. An animal is placed into a compartment and it has to either remain in that compartment to avoid a mild shock (passive avoidance) or go to another compartment (active avoidance, or escape-directed behavior) to avoid the aversive stimulus. Overall, conditioned tests provide less between-subject baseline variability than unconditioned response tests, but most conditioned assays require extensive training and the use of additional groups for controlling potential differences in learning and memory.

1.4 ANXIETY: CONTINUOUS EXPRESSION OF NORMAL HUMAN PERSONALITY TRAITS

It has long been proposed that the underlying structure of normal adaptive traits and the maladaptive personality traits of anxiety are the same [22]. Analysis of normal personality traits by NEO-PI in persons with psychiatrist-ascertained anxiety disorders in a general population showed an association of high neuroticism with lifetime anxiety disorders [simple phobia, social phobia, agoraphobia, panic disorder, obsessive-compulsive disorder (OCD), and generalized anxiety disorder] (Fig. 1.1). Social phobia and agoraphobia were also associated with low extroversion, and OCD was associated with high openness to experience [43]. In the Cloninger model, anxiety incorporates many aspects of harm avoidance [22]. Autonomic arousal, an integral part of neuroticism, is also a characteristic of anxiety disorders and is manifested as tachycardia, increased blood pressure, and elevated core temperature [44].

Recent genetic studies further support the notion that anxiety is the continuous expression of certain personality traits. For example, neuroticism/harm avoidance share a common genetic variant with susceptibility to anxiety disorders. Lesch et al. demonstrated that a functional 5-HTT promoter polymorphism is associated with the NEO-PI-R factor neuroticism and harm avoidance of the Cloninger model [9]. Extension of these genetic studies to anxiety disorders by the same authors showed no differences in 5-HTT genotype distribution between anxiety patients and comparison subjects, but among anxiety patients, carriers of a specific 5-HTT allele exhibited higher neuroticism scores than noncarriers [45]. Over 20 other studies investigated this association, and recent meta-analyses of these studies found a small but significant association between 5-HTT polymorphism and in some but not all measures of neuroticism/anxiety [46]. These studies remind us of the multifactorial nature of anxiety and that individual genes have only a small contribution to the clinical phenotype.

1.4.1 Anxiety Disorders

In the United States, anxiety disorders are most often defined and diagnosed according to a categorical system established by the **Diagnostic and Statistical Manual of Psychiatric Disorders**, currently in its fourth edition (DSM-IV) [5]. The DSM-IV sets the boundary at which a particular level of emotionality becomes an anxiety disorder—a level often based on the number and duration of observable symptoms of anxiety. This categorical model of anxiety, although necessary for the clinical diagnosis of anxiety disorders, is far from being reflective of the biological

nature of emotional states. Emotionality and anxiety are more realistically illustrated through a dimensional model that encompasses a continuum of various measures. The subjectivity of diagnosis is further illustrated by the marked differences in the diagnostic criteria of generalized anxiety disorder between the DSM-IV [5] and the ICD-10 Classification of Mental and Behavioral Disorders [47]. Although a core group of symptoms is identical in the two systems, DSM-IV relates these symptoms to vigilance while ICD-10 emphasizes the importance of autonomic arousal/hyperactivity. For the purpose of the present review, a quick overview of the DSM-IV classifications of anxiety disorders will be presented, while further discussions on different diagnostic criteria of DSM-IV and ICD-10 can be found elsewhere [48, 49].

The DSM-IV provides diagnostic criteria for a number of anxiety disorders, including panic disorder, specific and social phobias, OCD, posttraumatic stress disorder (PTSD), and generalized anxiety disorder [5, 50]. Individuals suffering from panic disorder experience recurrent and unexpected panic attacks which lead to discrete periods of intense fear and/or discomfort. Panic attacks are characterized by increased autonomic responses, including increased heart and breathing rates, sweating, nausea, abdominal distress, chills or hot flashes, and lightheadedness. Panic disorder may include agoraphobia, defined as the avoidance of places or situations in which escape may be difficult or embarrassing in the event of a panic attack. Specific and social phobias are marked by persistent fear of either clearly discernible objects or situations or potentially embarrassing social or performance situations, respectively. Exposure to the phobic stimulus almost invariably leads to heightened anxiety that may be expressed as a panic attack. Phobic stimuli are most often actively avoided. OCD features recurrent obsessions and compulsions severe enough to interfere with everyday life. Obsessions are described as persistent and inappropriate anxiogenic ideas, thoughts, or impulses that are unrelated to a real-life problem. Individuals suffering from OCD reduce obsession-induced anxiety by performing repetitive behaviors known as compulsions. These excessive and stereotypic behaviors or mental acts are not realistically connected with what they are designed to neutralize (i.e., washing and cleaning, counting, checking, and rearranging, etc.). PTSD can develop following a traumatic event involving feelings of intense fear, helplessness, or horror (e.g., military combat, rape, assault, and serious accident). Patients experience distressing recollection of the event, numbing of general responsiveness, and persistent arousal. They make deliberate and persistent efforts to avoid trauma-associated stimuli. Finally, generalized anxiety disorder is characterized by persistent (over six months) and excessive worry, inability to control worry, muscle tension, irritability, and sleep disturbance that are not necessarily related to a specific threatening situation. Many individuals also experience somatic symptoms (dry mouth, sweating, nausea, urinary frequency) that are reminiscent of certain symptoms of panic attacks [5].

1.4.2 Anxiety-Like Behavior in Animals

Studying rodent behavior in various anxiety-related test paradigms (see Section 1.3.2) reveals variation in emotionality in these species [51–53]. This includes variability in avoidance of aversive environment (Av), activity/behavioral inhibition in highly or moderately threatening situations (Ac), and autonomic arousal (Aa)

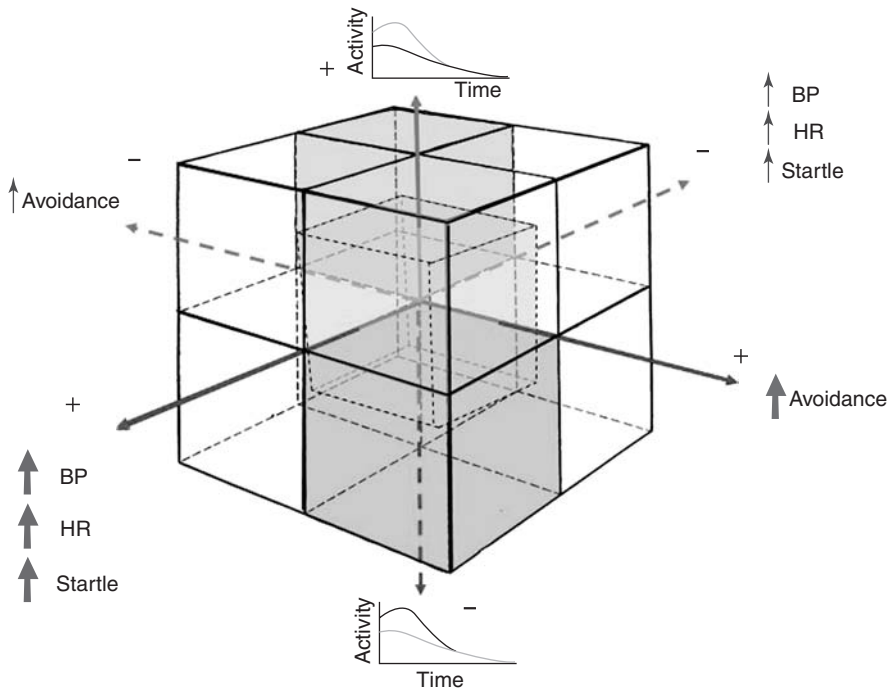


Figure 1.3 The three dimensions (AvAcAa) of emotionality in rodents in three-dimensional representation. Avoidance (Av) is plotted on the x axis, the positive spectrum representing increased levels of avoidance in stress-inducing environments or following stress-inducing stimuli and the negative spectrum representing attenuation of avoidance or even increased risk-taking behavior. Activity (Ac) as a response to stress and fear is plotted on the y axis, with the positive spectrum corresponding to increased levels of activity in a moderate to highly stressful environment. Following habituation, the activity is not different. Decreased activity in such an environment would be plotted in the negative spectrum of the y axis. Finally, the z axis is used for autonomic arousal (Aa) elicited by fearful or stressful stimuli, including increased heart rate, increased blood pressure, heightened levels of muscle tension (as measured by the startle response), defecation, and urination. A positive or negative deviation from the normal level of autonomic arousal can be represented by the positive and negative spectra of this axis, respectively. The normal range of these measures in a population is represented by the gray cube. Increased anxiety-like behavior can be conceptualized as increased avoidance, reduced activity, and increased arousal beyond the normal range of variations, denoted by the red area of the cube. Reduced anxiety-like behavior or increased novelty-seeking/risk-taking behavior is characterized by attenuation of avoidance, increased activity, and reduced autonomic arousal, highlighted by the blue area of the cube. (See color insert.)

(Fig. 1.2). Figure 1.3 displays these three basic characteristics as dimensions that together determine the degree of emotionality (see gray box for the range of normal variation of the three dimensions).

Many selectively bred and genetically modified mouse/rat strains show significant deviations from the normal variability of these dimensions. Once measures of behavior in mutant rodents exceed the threshold of variance of the normal/control

population (increased avoidance, reduced activity, and increased autonomic arousal), the resulting condition can be conceptualized as anxiety-like and similar to anxiety disorders in humans (Fig. 1.3; see red area of the cube). Emotionality can also be decreased (attenuation of avoidance, increased activity, and attenuated autonomic arousal) in a novel environment (Fig. 1.3; see blue area of the cube). Indeed, individual animals with higher and lower emotionality have been selected from a population and bred selectively to obtain strains characterized with high and low anxiety-like behavior. The Maudsley reactive inbred rat strain shows a stable and reproducible deficit in exploratory behavior as compared to the Maudsley non-reactive strain [54]. A similar breeding strategy based on behavior in the EPM test (open arm entries and time) resulted in the high-anxiety-related behavior (HAB) and low-anxiety-related behavior (LAB) rat lines [55]. These differences in behavior presumably reflect contributions from multiple genetic loci. Since generating induced mutations in mice has become routine, numerous mutant strains with either increased or decreased anxiety-like phenotype have been identified (see detailed description of these lines in Section 1.8.2).

Mutant mice with increased emotionality/fear reactions can be used as models of anxiety, and it is important to determine if they have construct and face validity. The criterion of construct validity requires that the rationale used to form the animal model is based on the etiology and the biological factors of anxiety. Construct validity criteria are difficult to fulfill because factors underlying the human disorders are largely unknown. However, in a few cases, the animal model has a genetic defect similar to that identified in anxiety disorders. For example, reduced expression of the 5-HT_{1A} receptor has been repeatedly shown in anxiety disorders and mice heterozygous for the inactivated 5-HT_{1A} receptor have an increased anxiety-like phenotype (see Sections 1.6 and 1.10).

Face validity represents a similarity in the physiological and behavioral measures observed in humans and in the animal model. As with construct validity, some animal models meet this criterion more easily than others. Physiological expressions of fear as well as anticipation of fear are comparable across species as they include easily quantifiable autonomic or endocrine responses such as increases in heart rate, blood pressure, body temperature, and muscle tension or changes in plasma corticosterone.

Predictive validity refers to the sensitivity of the model to clinically effective pharmacotherapeutic drugs. Benzodiazepines, for example, are commonly used in the treatment of anxiety; hence, a proposed animal model with predictive validity should show decreased measures of anxiety following benzodiazepine administration. In contrast, anxiogenic compounds should produce the opposite in physiological and behavioral measures. In addition, compounds with no effect in the clinic should not alter these measures in an animal model. Although predictive validity is an essential criterion for an animal model of anxiety in preclinical research, it has less relevance in studies focusing on the pathogenesis of anxiety disorders. Indeed, sensitivity to anxiolytics such as benzodiazepines varies in the population [56, 57]. For example, it has been shown that subjects high in neuroticism [58] and panic disorder patients [59–61] are less sensitive to benzodiazepines, with true benzodiazepine treatment resistance occurring in up to 24% of panic patients [62]. A similar difference in drug response can also be seen in certain animal models of anxiety such as the 5-HT_{1A} receptor-deficient mouse strains on various genetic backgrounds [38].

1.5 FEAR/ANXIETY CIRCUITS

1.5.1 Brain Regions Related to Anxiety Disorders

Anxiety is an emotion involving a complex interaction among many interconnected brain regions, with each component playing a specific role [63]. Most of these brain regions are part of the basic fear network, which is comprised of the prefrontal cortex, hippocampus, thalamus, and amygdala and its projections to brain regions responsible for coordinating the behavioral, autonomic, and endocrine response to fear (i.e., ventral tegmental area, locus ceruleus, dorsal motor nucleus of the vagus, nucleus ambiguus, lateral hypothalamus, paraventricular nucleus of the hypothalamus, etc.). Imaging technologies such as positron emission tomography (PET), magnetic resonance imaging (MRI), and functional MRI (fMRI) have made a large impact on elucidating the roles of various fear pathway structures in anxiety disorders. One of the best characterized limbic structures for its role in processing fear-related stimuli is the amygdala [64–66]. Furthermore, neuroimaging studies have shown that abnormal amygdala function is involved in anxiety disorders. Excess amygdala activation has been observed in PTSD patients in response to stimuli reminiscent of the traumatic event [67, 68] as well as in specific phobia patients when exposed to a phobia-related stimulus [69]. A volumetric MRI study revealed a significantly lower bilateral amygdala volume in panic disorder patients compared to individuals in the healthy control group [70]. Abnormal amygdala volume is not specific to panic disorder, as reduced amygdala volume has also been observed in patients suffering from OCD [71]. In contrast, larger right amygdala volume was measured in generalized anxiety disorder patients [72]. However, this particular study was performed in children; thus age, in addition to different anxiety diagnosis, may explain the contradicting results. Interestingly, the same cohort of children was later followed up with an fMRI study in which an exaggerated right amygdala response to fearful faces was observed in generalized anxiety disorder patients but not in healthy children. These results are suggestive of a relationship between structure and function and indicate that hyperactivity of the amygdala may be a characteristic feature of some anxiety disorders [73].

In addition to the hyperactivity of the amygdala, a number of neuroimaging studies have reported functional abnormalities in other fear pathway substrates in anxiety disorder patients. For example, increased levels of activity were found in the orbitofrontal cortex, hippocampus, and anterior and posterior cingulate in response to directed imagery of strongly emotional personal experiences in subjects suffering from panic disorder compared to healthy individuals [74]. Exaggerated activation of the orbitofrontal cortex has also been documented in specific phobia patients [69]. In contrast, the anterior cingulate gyrus showed lower levels of activity in PTSD during exposure to emotional stimuli by several groups [72, 75–77]. The anterior cingulate abnormality, together with the observed hyperactivity of the amygdala, has been incorporated into a neuroanatomical model of PTSD. Medial prefrontal structures, including the cingulate cortex, are thought to inhibit the activity of brain regions involved in fear responses, and therefore a hypoactive medial prefrontal cortex would fail to inhibit the amygdala in this model of PTSD [77, 78].

The hippocampus has also been extensively studied by neuroimaging techniques. Volumetric imaging studies that have been performed on PTSD patients have yielded

conflicting results with regard to hippocampal size. Some have found no differences in hippocampal volume between PTSD patients and non-trauma-exposed controls [79, 80], while others have documented either unilateral or bilateral reduction in hippocampal volume in PTSD patients [81–84]. A fundamental problem with most imaging studies is that the correlation between size of a neural substrate and the disorder in which it is documented may not be causal. In an attempt to address this issue, Wignall et al. [83] measured the hippocampal volume of recent-onset PTSD patients and found a decrease in right-sided hippocampal volume. Although the authors could not exclude the possibility that the hippocampal damage occurred during the time between the traumatic event and the onset of PTSD (mean of 158 days), they leaned toward the interpretation that smaller hippocampal volumes predispose individuals to the development of PTSD. A similar, yet longitudinal MRI study, however, showed that survivors of traumatic events who developed PTSD had no differences in hippocampal volumes at one week and at six months following the trauma when compared to trauma survivors that did not develop PTSD [80]. This particular controversy was somewhat abated by a study that found that monozygotic twins of PTSD combat veterans who themselves were not exposed to combat showed comparable hippocampal volumes to their combat-exposed brothers and that the hippocampi of these twins were significantly smaller than those of both combat veterans without PTSD and their non-combat-exposed twins [81]. These results indicate that a smaller hippocampal volume is a pre existing PTSD predisposing factor rather than a product of the disorder. A reduced hippocampal volume is not, however, a prerequisite for the development of the disorder.

Certain dopaminergic substrates, particularly the ventral striatum, have been found to be both larger in volume [85] and functionally hyperactive in patients suffering from OCD. Other hyperactive regions documented include orbitofrontal cortex, caudate, thalamus, and the anterior cingulate cortex [85, 86]. Based on these data the prevailing hypothesis of OCD pathogenesis proposes that OCD symptoms are mediated in part by a defect in the orbitofrontal-subcortical circuits.

1.5.2 Brain Regions Related to Emotionality/Anxiety-Like Behavior in Animals

Brain regions involved in fear and emotionality in animals are largely the same as those implicated in anxiety disorders (Fig. 1.4). The amygdala has a central importance in the acquisition, retention, and expression of conditioned fear [87–89]. The amygdala seems to function as an emotional/cognitive interface receiving sensory information via projections from the cortex and the thalamus. Outputs from the amygdala to the frontal cortex are related to the conscious perception of fear while outputs to the locus ceruleus, hypothalamus, periaqueductal grey, and striatum mediate autonomic, neuroendocrine, and skeletal-motor responses associated with fear and anxiety.

Although it is widely accepted that the hippocampus plays an important role in certain forms of learning and memory, recent studies have shown that the hippocampus is also involved in fear and emotionality. Interestingly, ventral hippocampal lesions affect anxiety while dorsal lesions result in defects in spatial learning. For example, cytotoxic lesions of the ventral hippocampus resulted in reduced aversion in the center of the open field, reduced freezing after footshock, and reduced inhibition in novelty-suppressed feeding [90]. Also, lesion of the ventral but not dorsal

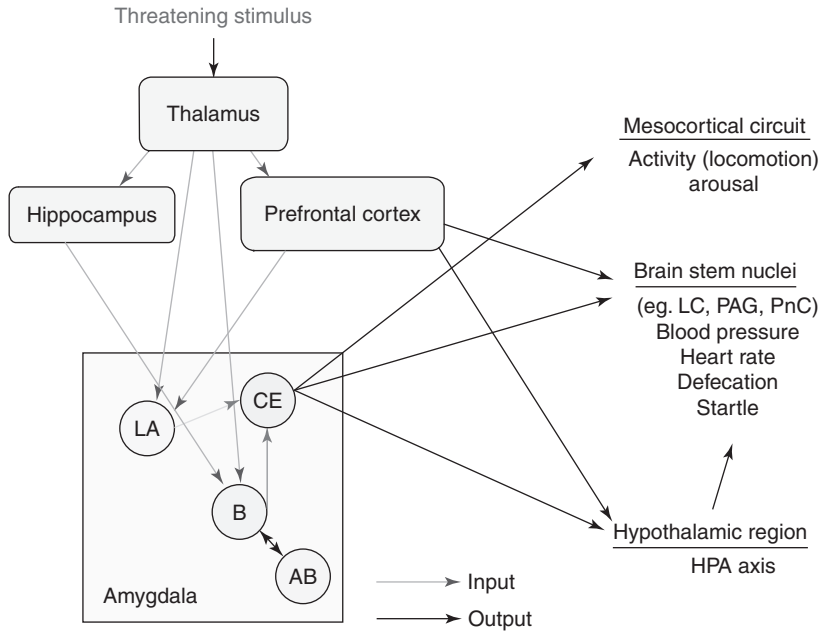


Figure 1.4 Brain regions involved in processing fear, stress, and emotionality in animals. Threatening stimuli are received and processed by brain regions such as the thalamus, hippocampus, prefrontal cortex (PFC), and amygdala. The amygdala sends projections to a number of target regions. These include brain stem nuclei such as the locus ceruleus (LC), periaqueductal gray (PAG), and pontine nucleus caudalis (PnC), which mediate various forms of autonomic arousal. The amygdala is also connected to the mesocortical circuit that mediates arousal and activity (as measured by locomotion in mice) and the hypothalamic region which controls glucocorticoid levels through the hypothalamic–pituitary–adrenal (HPA) axis. The PFC, which is involved in executive functions such as attention, also sends projections directly to the brain stem and the hypothalamus. (See color insert.)

hippocampus increased open-arm exploration in EPM [91, 92]. The septohippocampal system has been identified as being essential for the sensory processing of stimuli based on novelty and punishment [93]. Hippocampus has also been implicated in contextual fear conditioning [94].

Forebrain structures, including the medial prefrontal cortex (MPFC) and septum, are connected to the limbic system and their dysfunction has also been found in anxiety. Also, several studies have shown that lesions (cytotoxic and transection) of the MPFC inhibit fear-related behavior in rats [95–98]. These data indicate that MPFC promotes anxiety-like behavior. Finally, brain stem nuclei are important in the regulation of arousal. Of particular importance in anxiety are the noradrenergic locus ceruleus and the serotonergic raphe nuclei [99, 100].

1.6 NEUROTRANSMITTER SYSTEMS AND NEURONAL MESSENGERS IMPLICATED IN ANXIETY AND ANXIETY-LIKE BEHAVIOR

Traditionally, anxiety disorders have been viewed as disturbances in neurotransmitters, including γ -aminobutyric acid (GABA), 5-HT, norepinephrine (NE), dopamine

(DA), and neuropeptides such as corticotropin-releasing hormone (CRH), cholecystokinin (CCK), and neuropeptide Y (NPY). Many of these neurotransmitters and their receptors have been identified as sites of action for anxiolytic drugs. However, neuronal messengers other than neurotransmitters such as cytokines have recently been implicated in anxiety. Here we summarize the relevant pharmacological data while a later section covers the pertinent genetic studies.

Alterations in GABA_A receptor function have long been implicated in anxiety disorders. For example, a deficit in GABA_A receptors has been identified in the hippocampus and parahippocampus of patients suffering from panic disorder and generalized anxiety disorder [101–103]. Furthermore, GABA_A receptor antagonists can elicit anxiety in patients with panic disorder, thereby mimicking a functional deficit of GABA_A receptors [104]. The GABA_A receptor is a pentameric ion channel typically composed of 2 α (α_{1-6}), 2 β (β_{1-3}), and 1 γ (γ_{1-3}) subunits [105, 106], and animal studies suggest that alterations in specific GABA_A receptor subunits are associated with certain forms of anxiety, such as withdrawal-induced anxiety [107, 108]. GABA_A receptor subunits have an especially important relevance in terms of the anxiolytic effect of benzodiazepines [109–111]. Classical benzodiazepines exert their effects by binding to multiple subtypes of GABA_A receptor, the predominant subtypes in the brain being those that contain $\alpha_{1,2,3,5}$ subunits. A recent report using receptor subtype–preferring compounds in nonhuman primate models concluded that α_1 subunits containing receptors do not play a key role in the anxiolytic and muscle-relaxant properties of benzodiazepine-type drugs; instead, these effects involve $\alpha_{2,3,5}$ subunits containing GABA_A receptors [112]. Animal models have recently also been used to determine the GABA_A receptor subtype involved in the anxiolytic action of benzodiazepines (see description of these animals in Section 1.10).

Although lesion of 5-HT neurons in animals suggests a role for 5-HT in the control of anxiety states [113], the evidence for this notion is both conflicting and controversial. On the other hand, pharmacological manipulation of either the 5-HTT or the 5-HT_{1A} receptor can clearly alter 5-HT neurotransmission and anxiety. The level of 5-HT is regulated by both the 5-HTT and the 5-HT_{1A} autoreceptor (in the serotonergic raphe nuclei) [114, 115]. Inhibiting 5-HTT by selective serotonin reuptake inhibitors (SSRIs) has been shown to be very effective in certain anxiety disorders [114, 115]. Also, partial 5-HT_{1A} receptor agonists such as buspirone have an anxiolytic effect [116]. Recently, animals with genetic modifications have significantly contributed to our understanding of the 5-HT system and the possible role of various 5-HT receptors in anxiety (see Section 1.10).

The role of NE in anxiety is based on its well-known involvement in stress reaction. Stress provokes and aggravates anxiety by increasing catecholamine release via the sympathoadrenal system in the periphery. In addition, NE neurons in the locus ceruleus play a critical role in the body's response to alarm and threat. NE is believed to play an especially important role in anxiety disorders, such as panic disorder and PTSD [117, 118].

DA is mostly known as a mediator of reward and locomotor activity. However, these processes are also fundamental in personality traits and emotionality (Figs. 1.1–3), and the pharmacological manipulation of DA receptors have been reported to modulate anxiety-related behaviors. Specifically, agonists and

antagonists for the DA D₂ class of receptors (which includes D₂, D₃, and D₄ subtypes) have anxiogenic and anxiolytic properties, respectively [119–121].

A number of neuropeptides have been implicated in anxiety and have been suggested as therapeutic targets [122]. The stress response is mediated partly by the activation of CRH. CRH is produced in the hypothalamus (H), leading to the secretion of the adrenocorticotropin hormone (ACTH) from the pituitary (P), which in turn causes an increase in the synthesis and release of glucocorticoids from the adrenal glands (A) (HPA axis). The activation of the HPA axis is also involved in stress-related psychopathology such as anxiety disorders [123–125]. The maladaptive effects of chronic stress on the HPA axis have been extensively studied in both preclinical and clinical settings, and since a number of excellent reviews are available, this topic is not discussed further here [125–129]. In addition to the activation of the HPA axis and the consecutive release of the stress hormones, CRH is present outside of the hypothalamus where it is believed to participate in stress response [130]. Central administration of CRH in rodents produces behavioral effects that correlate with a state of anxiety such as reduced exploration in a novel environment or enhanced fear response [131–134]. Preclinical studies strongly implicate a role for central CRH, probably via the central noradrenergic systems, in the pathophysiology of certain anxiety disorders [125].

Glucocorticoids (corticosterone in rodents and cortisol in humans), the final effectors modulating the physiological response to stress, act via two receptor subtypes: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) [135]. GRs are also the main regulators of a negative-feedback circuit that regulates the HPA axis following stress. Activation of GRs in the pituitary, hypothalamus, hippocampus, and frontal cortex decreases CRH gene expression, leading to a decrease in CRH release and the suppression of the stress-induced endocrine response [136, 137].

Another neuropeptide, NPY, has also been suggested to be involved in the clinical symptoms of anxiety [138]. In rats, central administration of NPY produces effects similar to that of anxiolytic drugs [139] whereas specific inhibition of the NPY-1 receptor by antisense oligonucleotide resulted in an increased anxiety-like behavior [140].

During the last few years, CCK has emerged as an important polypeptide in the central nervous system (CNS). There are several lines of evidence for a role of CCK in anxiety and panic attacks, and data also indicate that specific agonists to brain CCK(2) receptors produce anxiogenic-like effects while CCK(2) antagonists elicit anxiolytic-like responses [141–144].

Substance P has also been suggested to have a modulatory role in anxiety [145]. Substance P is released in response to aversive stimuli [146] and its administration in animal models elicits both anxiogenic and anxiolytic activity, depending on the dose and the specific brain region [122]. The receptor for substance P is the G-protein-coupled tachykinin NK-1 receptor which is expressed in brain areas associated with fear and anxiety [147]. Increasing numbers of reports indicate that specific antagonists of NK-1 receptors produce anxiolytic effects [148].

Although cytokines are not neurotransmitters and their primary role is in the immune system, several lines of evidence indicate that interleukin (IL) 1 β , interleukin-6, and tumor necrosis factor (TNF) α modulate anxiety and mood [149].

Specifically, these proinflammatory cytokines elicit symptoms of anxiety/depression that may be attenuated by chronic antidepressant treatment. Also, immunotherapy using IL-2 or interferon (IFN) α , promotes depressive/anxiety symptoms [149]. Interestingly, the effects of cytokines are exacerbated by stressors, and chronic cytokine elevations may act synergistically with stressors [149].

1.7 GENETIC SUSCEPTIBILITY TO ANXIETY DISORDERS

A number of studies have sought to identify chromosomal regions and genes relevant to anxiety disorders. Although the results of linkage and association studies are inconsistent so far (see detailed description of these studies in [150–152]), candidate gene studies have yielded more consistent data. In several studies, a relatively small but significant increase in neuroticism was found in individuals who carry the *s/s* (short promoter repeat) alleles of the 5-HTT as compared to individuals with *s/l* (long) or *l/l* alleles [9, 153]. The *s* allele is associated with decreased transporter activity. Over 20 other studies extended this association to psychopathology, but not all found evidence for an association between 5-HTT polymorphism and anxiety [45]. However, recent meta-analyses of these studies found a moderate but significant association between 5-HTT polymorphism and NEO neuroticism [46] and TPQ (tridimensional personality questionnaire) harm avoidance [154]. The association of decreased transporter activity with anxiety is a rather surprising finding because pharmacological inhibition of the 5-HTT by SSRIs reproducibly results in an anxiolytic effect. However, the genetically determined reduction in 5-HTT activity in patients is present from early prenatal life and may affect brain development leading to anxiety in later life. Consistent with this notion, pharmacological inhibition of 5-HTT in early postnatal life in mice (which corresponds to late prenatal life in human) resulted in increased anxiety-like behavior in later life [155]. In summary, these data suggest that genetic or pharmacological reduction of transporter activity during brain development can lead to increased anxiety in adult life.

The 5-HT_{1A} receptor (5-HT_{1A}R) has also been implicated in anxiety because reduced receptor levels were detected in the anterior cingulate, posterior cingulate, and raphe by positron tomography in patients with panic disorder [156]. These recent data complement previous reports that showed a deficit in the 5-HT_{1A}R in PTSD and panic disorder patients [157–160]. However, no specific 5-HT_{1A}R allele has been associated with anxiety disorders (a promoter polymorphism, on the other hand, has been linked to major depression and suicide [159]).

Although polymorphism in BDNF has primarily been studied in depressive disorder, the *val* allele of the Val66Met substitution polymorphism has recently been shown to be associated with higher mean neuroticism scores in the NEO- five factor inventory (NEO-FFI) in healthy subjects [161]. In another study the self-ratable state-trait anxiety inventory (Spielberger state-trait anxiety inventory) score, which allows anxiety to be quantified as a comparatively stable personality trait, showed a higher level of anxiety in Val/Val compared to Val/Met and Met/Met genotypes [11, 162]. These are surprising findings since it is the *met* allele that is hypofunctional (as a result of alterations in BDNF trafficking and secretion [163])

and because animal studies clearly show that genetic inactivation of BDNF results in anxiety (see Section 1.9).

1.8 GENETIC BASE OF ANXIETY-LIKE BEHAVIOR IN MICE

1.8.1 QTL Studies

QTL analysis of F2 hybrids of two strains of mice (A/J and C57BL/6J) that differ markedly in thigmotaxis and light-to-dark (LD) transition behaviors showed a linkage of LD to chromosome 10 (near D10Mit237; LOD of 9.3) and suggestive QTLs (LOD > 2.8) at chromosomes 6, 15, 19, and X [14]. In the open field, suggestive QTLs were mapped to chromosomes 6 and 14 [13]. These data indicate a lack of shared QTLs of fear/anxiety-associated behavior in various experimental paradigms (avoidance in LD and open field). Another group using multiple measures of avoidance and autonomic arousal found that the various measures are mapped to the same or nearby chromosomal location(s) [12, 15]. These studies used two relatively closely related mouse lines bred for differential anxiety-like behavior; thus a relatively small subset of genes may have changed during breeding. In contrast, the study that concluded a lack of shared QTLs in anxiety-like behavior [13] utilized the more distantly related A/J and C57BL/6J mice which presumably carried different anxiety-related alleles in multiple loci. This study may be easier to extrapolate to human populations characterized by a high degree of heterogeneity. So far, no genes have been identified in anxiety-related QTLs. Since QTLs are in the range of 10–30 cM, a region containing hundreds of genes, identification of linked genes within QTLs is difficult.

1.8.2 Anxiety-Like Behavior in Genetically Altered Mice

Recently, it has become possible to inactivate specific genes routinely in the mouse, and a large number of knockout strains have been generated. Many of the targeted genes have been implicated in anxiety, and the corresponding knockout strains have regularly showed behavioral abnormalities in anxiety-related tests (Tables 1.2 and 1.3). Beyond the mouse strains with inactivated “candidate” anxiety genes, anxiety-like behaviors were sometimes seen in mice with genetic inactivation in genes not obviously related to anxiety. These genes include intracellular signaling molecules and regulators of transcription/translation (Table 1.4). The association of these genes with anxiety-like phenotype indicates that anxiety is not limited to abnormalities of the neurotransmitter systems but can also be related to gene regulatory processes. Analysis of the genomic position of these genes shows that they are distributed throughout many chromosomes with no obvious clustering at any locus (Fig. 1.5).

One caveat of the analysis of anxiety-related knockout mouse strains is that the behavioral phenotypes are not always robust and are sometimes even questionable. Moreover, many variables can alter the interpretation of anxiety-related behavioral tests and tests are not standardized across laboratories and environmental factors. Furthermore, anxiety-like behavior may be part of a complex phenotype and secondary to major developmental or neuroanatomical defects. We limited our

TABLE 1.2 Mice with Genetic Alteration of Neuronal Messengers Exhibiting Altered Anxiety Levels

Targeted Gene/Protein	Behavioral Test	Avoidance (Av)	Activity (Ac)	Arousal (Aa)	Anxiety	Chr	Reference
GAD65 KO	OF, EZM	+	—	n/a	↑	chr2 qA3 (1)	[164]
COMT KO	L/D	+(F only)	— (F only)	n/a	↑	chr16 qA3 (2)	[168]
NET KO	OF	n/a	—	n/a	?	chr8 qC5 (3)	[169]
5-HTT KO	OF, EZM	+	—	+	↑	chr11 qB5 (4)	[171]
	OF, EPM, NSF, AA, FPS	+/-	0	n/a	↑/0	—	[172]
CRH overexpressing	OF, EPM	+	—	n/a	↑	chr3 qA2 (5)	[173, 174]
CRH KO	EPM, OF, and other	0	0	—	0	—	[175]
CRH-BP overexpressing	OF, EPM	—	+	0	↓	chr13 qD1 (6)	[180]
CRH-BP KO	EPM, OF and other	+	—	0	↑	—	[179]
NPY KO	OF, AS, EPM, PA	0	—	+	↑	chr6 qB2.3 (7)	[181]
ProEnkephalin KO	OF, EZM	+	—	n/a	↑	chr4 qA1 (8)	[182]
OFQ/N KO	OF, EPM, L/D	+	—	+	↑	chr14 qD1 (9)	[184]
BDNF cond KO	OF, EPM, L/D	+	n/a	+	↑	—	[185]
TNF α overexpressing	OF, L/D	+	+/-	n/a	↑	chr2 qE3 (10)	[190]
TNF α KO	L/D	+	—	n/a	↑	chr16 qA1 (11)	[192]
Interferon γ KO	OF, EPM	+	0	0	↑(?)	—	[194]
	OF, EPM, PA	+	—	+	↑	chr10 qD2 (12)	[195]

Notes: Av/AcAa shown as decreased (—), increased (+), or not different (0) from wild-type (WT) controls. n/a = data not obtained by investigator. F = females; Chr = chromosome location and assigned number corresponding to illustration in Figure 1.5; OF = open-field exploration; KO = knockout; EZM = elevated-zero maze; L/D = light–dark box; EPM = elevated-plus maze; NSF = novelty-suppressed feeding; AA = active avoidance; FPS = fear-potentiated startle; AS = acoustic startle; PA = passive avoidance; GAD65 = glutamic acid decarboxylase, 65-kD isoform; COMT = catechol-O-methyl transferase; NET = norepinephrine transporter; 5-HTT = serotonin transporter; CRH = corticotropin-releasing hormone; CRH-BP = CRH binding protein; NPY = neuropeptide Y; OFQ/N = orphanin FQ/nociceptin; BDNF = brain-derived neurotrophic factor; TNF α = tumor necrosis factor α .

TABLE 1.3 Mice with Genetic Alteration of Receptors and Other Cell Membrane-Associated Proteins Exhibiting Altered Anxiety Levels

Targeted Gene/Protein	Behavioral Test	Avoidance (Av)	Activity (Ac)	Arousal (Aa)	Anxiety	Chr	Reference
GABA _A R: γ_2 ^{+/−}	EPM, OF, L/D, PA, FC	+	−	n/a	↑	chr11 qA5 (13)	[196]
5HT _{1A} R KO	EPM, OF, EMZ and other	+	−	+	↑	chr13 qD1 (14)	[207–209]
5HT _{1B} R KO	OF, EPM, NSF	−	+	n/a	↓	chr9 (15)	[220]
	EPM, FC, and other	0	0	n/a	0	—	[94]
CRH-R1 KO	L/D, EPM	−	0	−	↓	chr11 qE1 (16)	[224]
CRH-R2 KO	EPM, L/D, OF	+ / 0	0 / +	+	↑	chr6 qB3 (17)	[225, 226]
DA D ₃ KO	OF, EPM	−	+	n/a	↓	chr16 qB4 (18)	[229]
α_2A -AR KO	EPM, OF	+	−	n/a	↑	chr19 qD2 (19)	[234]
Adenosine A _{2a} KO	EPM, L/D	+	−	n/a	↑	chr10 qC1 (20)	[236]
nAChR α_7 KO	OF, L/D, AS, FC	0	+	0	0 / ↓	chr7 qB5 (21)	[243]
nAChR α_4 KO	EPM	+	−	n/a	↑	chr2 qH4 (22)	[244]
trkb overexpressing	EPM, L/D, FC	−	+	n/a	↓	chr13 qB2 (23)	[246]
NCAM KO	EPM, L/D	−	+	n/a	↓	chr9 qA5.3 (24)	[248]
L1 cond KO	EPM, OF	−	+	n/a	↓	chrX qA7.2 (25)	[249]
Cadherin 11 KO	EPM, AS, FC	−	+	−	↓	chr8 qD1 (26)	[250]
GIRK2 KO	EPM, L/D	−	+	n/a	↓	chr16qC4 (27)	[238]

Notes: Av/AcAa shown as decreased (−), increased (+), or not different (0) from wild-type (WT) controls. n/a = data not obtained by investigator. Chr = chromosome location and assigned number corresponding to illustration in Figure 1.5; OF = open-field exploration; KO = knockout; EZM = elevated-zero maze; L/D = light-dark box; EPM = elevated-plus maze; FC = fear conditioning; AS = acoustic startle; PA = passive avoidance; GABA_AR: γ_2 = γ -aminobutyric acid receptor A, γ_2 subunit; 5-HT_{1A/1B}R = serotonin 1A or 1B receptor; CRH-R1/R2 = CRH receptor 1 or 2; DA D₃ = dopamine D₃ receptor; α_2A -AR = α_2A -adrenergic receptor; nAChR $\alpha_7/4$ = nicotinic acetylcholine receptor α_7 or α_4 ; trkB = neurotrophin receptor tyrosine kinase B; NCAM = neural cell adhesion molecule; L1 = NCAM L1; GIRK2 = G-protein-coupled inwardly rectifying K⁺ channel 2.

TABLE 1.4 Mice with Genetic Alteration of Intracellular Signaling Molecules and Transcriptional Regulators Exhibiting Altered Anxiety Levels

Targeted Gene/Protein	Behavioral Test	Avoidance (Av)	Activity (Ac)	Arousal (Aa)	Anxiety	Chr	Reference
α CaMKII KO	FC, OF	—	n/a	—	↓	chr18 qE1 (28)	[252]
PKCy KO	EPM, L/D and other	—	+	n/a	↓	chr7 (29)	[255]
Fyn trk KO	L/D, PA	+	—	n/a	↑	chr10 qB1 (30)	[256]
NF-kB p50 KO	OF, EPM and other	—	+	n/a	↓	chr3 qG3 (31)	[260]
GR KO	L/D, EZM	—	+	0	↓	chr18 qB3 (32)	[263]
GR overexpressing	EPM, L/D	+	—	0	↑	—	[265]
VDR KO	OF, EPM, L/D	+	—	n/a	↑	chr15 qF4 (33)	[266]
CREB KO	OF, EZM, EPM, L/D	+	—	0	↑	chr1 qC2 (34)	[268]
CREM KO	EPM, OF, EZM, FC	—	+ / 0	n/a	↓	chr18 qA1 (35)	[270]

Notes: Av/Aa shown as decreased (—), increased (+), or not different (0) from wild-type (WT) controls. n/a = data not obtained by investigator; Chr = chromosome location and assigned number corresponding to illustration in Figure 1.5; OF = open-field exploration; KO = knockout; EZM = elevated-zero maze; L/D = light-dark box; EPM = elevated-plus maze; FC = fear conditioning; PA = passive avoidance; α CaMKII = α -calcium-calmodulin kinase II; PKCy = protein kinase C γ ; Fyn trk = Fyn tyrosine kinase; NF-kB = nuclear factor kB; GR = glucocorticoid receptor; VDR = nuclear vitamin D receptor; CREB = cAMP-responsive element binding protein; CREM = CAMP-responsive element modulator.

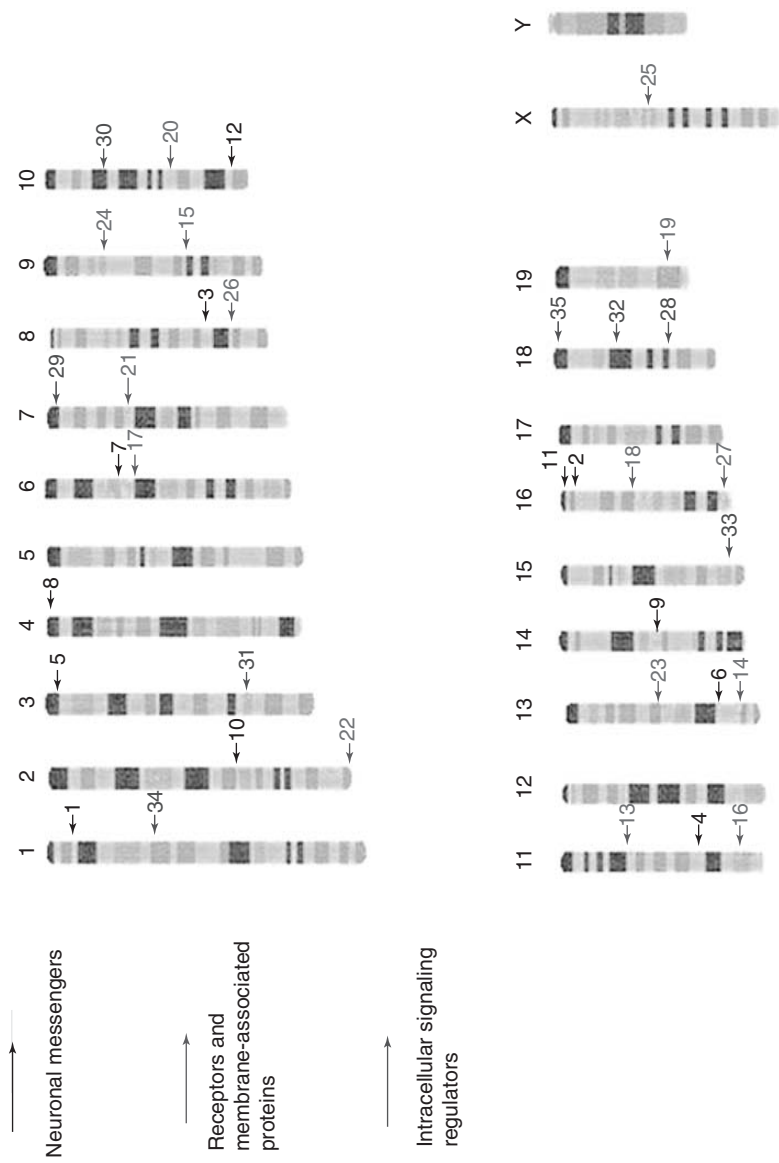


Figure 1.5 Chromosomal location of anxiety-related genes listed in Tables 1.2–4. (See color insert.)

analysis to mutant strains that have been generated and studied by multiple groups and to those that, although analyzed by a single laboratory, showed anxiety-related phenotypes in at least two independent behavior tests.

1.9 KNOCKOUT MICE WITH DISTURBANCES IN NEURONAL MESSENGERS EXHIBITING ALTERATIONS IN ANXIETY-LIKE BEHAVIOR

Classical neurotransmitters (GABA, NE, 5-HT) and neuropeptides have long been implicated in anxiety, so it was not surprising that inactivation of genes encoding enzymes responsible for the synthesis and metabolism of neurotransmitters or encoding neuropeptides alter anxiety levels (Table 1.2).

Glutamic acid decarboxylase (GAD) catalyzes the synthesis of GABA from glutamate and the genetic inactivation of the 65-kDa isoform of GAD (GAD65) results in anxiety-like behavior in mice [164]. Although GAD65 is responsible for the synthesis of a smaller pool of GABA than GAD67 [165, 166], it is associated with nerve terminals and synaptic vesicles and can be rapidly activated in times of high GABA demand. In GAD65^{-/-} tissues the overall GABA content is normal but K⁺-stimulated GABA release is reduced. Therefore, GAD65^{-/-} mice show no overt developmental phenotype but the more subtle anxiety-like behavioral phenotype [164] and increased seizure sensitivity [165]. In contrast, GAD67^{-/-} mice, although born at the expected frequency, die of severe cleft palate during the first morning after birth [166]. These data are consistent with reports that enhancing synaptic GABA levels, for example by GABA reuptake inhibitors, has an anxiolytic effect [167] and indicate that an appropriate level of synaptic GABA release is important for maintaining normal behavioral responses in anxiety-inducing situations.

The enzyme catechol-*O*-methyl transferase (COMT) is involved in the degradation of DA, NE, and epinephrine and its inactivation also leads to anxiety-like behavior [168]. Measurement of tissue catecholamine levels in COMT^{-/-} mice showed a specific increase in DA levels with no change in NE or 5-HT levels. Furthermore, this increase in DA seems to be restricted to the frontal cortex. Although the increased DA levels were evident in both males and females, an increased anxiety behavior was observed only in females.

Genetic inactivation of the NE transporter (NET), as expected, results in a significant increase of extracellular levels of NE [169]. These mice show increased activity in the open field that would be consistent with a reduced level of anxiety. However, no data are available regarding avoidance and autonomic arousal of these mice and hyperactivity can arouse independently of a change in anxiety.

Since 5-HTT (*s/s* genotype) has been identified as a susceptibility gene for anxiety (see Section 1.7), it was expected that mice with an inactivated copy of the corresponding gene would show elevated levels of anxiety. Initial studies indicated that knockout mice have no obvious behavioral phenotype even if 5-HTT binding sites were completely absent in these animals [170]. However, a later study indicated an increased anxiety-like phenotype in 5-HTT knockout mice which was more pronounced in females [171]. A more recent analysis of these mice found no differences in anxiety-related behaviors in the open-field and EPM tests, but an increase was seen in latency to feed in a novel environment [172]. Lack of a reproducible and robust anxiety-like phenotype in 5-HTT knockout mice raises the

question of how a partial reduction in 5-HTT activity in humans (*s/s* genotype) can be associated with elevated levels of neuroticism and anxiety.

Pharmacological experiments indicate the involvement of neuropeptides in the pathogenesis of anxiety, and this notion has been further supported by genetically modified mouse strains. An anxiety-like phenotype has been described in transgenic mice overexpressing CRH [173, 174]. However, mice with a deleted CRH gene did not differ from wild-type animals in anxiety-related behaviors even if they had significantly decreased basal corticosterone levels [175, 176]. One possible explanation is the redundancy in the central CRH system (e.g., urocortin). Consistent with this notion, stress-induced behavioral effects in CRH mutant mice could be reduced by the administration of a CRH antagonist [177]. Two independent groups have generated mice with a deletion of the urocortin gene [178], but only one study found behavioral abnormalities, namely an increased anxiety-like phenotype [178]. Finally, deletion of the CRH binding protein (BP), which normally binds and inactivates CRH, resulted in increased anxiety [179]. The authors hypothesized that the inactivation of CRH-BP may increase the “free” or unbound levels of CRH or urocortin, which results in anxiety. These data are also consistent with the reduced anxiety-like phenotype of mice constitutively overexpressing CRH-BP in the anterior pituitary gland [180].

Mice lacking the gene for NPY show a decrease in central area activity in the open field and an increased reactivity to acoustic startle [181]. However, no change in EPM was seen in NPY^{-/-} mice compared to controls, suggesting that the absence of the peptide results in a condition characterized by increased stress responsiveness rather than anxiety.

Apart from altered pain responses, preproenkephalin-deficient mice exhibit increased anxiety in the open field and EZM [182]. It is believed that the modulatory role of enkephalins on anxiety behavior may be mediated by the GABA system [183].

Consistent with the anxiolytic activity of orphanin FQ/nociceptin (OFQ/N) or selective synthetic agonists in rodents, OFQ/N knockout mice display increased anxiety in several anxiety-related tests and impaired adaptation to repeated stress [184, 185]. Increased plasma corticosterone levels and a failure to show stress adaptation of OFQ/N knockout mice may suggest that activation of the HPA axis contributes to the anxiety phenotype in these mice.

BDNF, a member of the family of neurotrophins, promotes the formation, maturation, and stabilization of both glutamatergic and GABAergic synapses during CNS development, and it therefore regulates the balance between excitatory and inhibitory transmission, a fundamental step in neural circuit formation [186, 187]. Although homozygote BDNF knockout mice die during the second postnatal week [180, 188], heterozygote or conditional knockout mice show signs of altered emotional behavior. The role of BDNF in emotional reactions is important because the *val* allele has been associated with predisposition to anxiety disorders [10, 11]. Heterozygote BDNF knockout mice showed a slower escape behavior in the learned helplessness paradigm after training as compared to wild-type mice [189]. However, this effect may have been due to reduced sensitivity to centrally mediated pain as BDNF is essential for the survival and maintenance of peripheral sensory neurons [180, 188]. On the other hand, conditional BDNF mutant mice have also shown other signs of anxiety-like behavior [190]. In these conditional knockout mice, BDNF was removed after birth when most neurons are postmitotic, suggesting that the

abnormal behaviors are related to neuronal maturation, survival, and/or plasticity rather than to the absence of BDNF during behavioral testing. Recently a mouse strain was generated in which the *val* allele was replaced by the *met* allele [190a]. Met substitution for Val in *BDNF* is a common polymorphism in humans associated with alterations in brain anatomy and memory. In agreement with association studies [10], BDNF(Met/Met) mice exhibited increased anxiety-related behaviors indicating that this variant predisposes to anxiety disorders.

In agreement with the anxiogenic effect of intracerebroventricularly administered TNF α in the EPM [191], transgenic mice overexpressing TNF α show less exploration in novel environment [192] and increased activity [193]. However, TNF α knockout mice have a similar behavior in the EPM [193, 194]; thus, the role of TNF in the regulation of emotionality is not clear. Lack of another cytokine, IFN γ , has been reported to cause an anxiety-like phenotype [195]. However, the expression of this phenotype was visible only in C57BL/6 but not in the BALB/c mouse strain, indicating that major genetic modifiers play a role in the manifestation of anxiety in these mice. IFN γ is involved in regulating the growth of axodendritic processes, raising the possibility that, similar to the BDNF knockout mice, a neurodevelopmental abnormality underlies the anxiety in these knockout mice.

1.10 KNOCKOUT MICE WITH DEFICITS IN NEUROTRANSMITTER RECEPTORS AND OTHER CYTOPLASMIC MEMBRANE-ASSOCIATED PROTEINS EXHIBITING ANXIETY-LIKE BEHAVIOR

Blocking GABA $_A$ receptor increases anxiety-like behavior and genetic inactivation of some of the subunit genes has a similar effect (Table 1.3). For example, heterozygote $\gamma_2^{+/-}$ mice have reduced numbers of GABA $_A$ receptors and display an anxiety phenotype [107, 196]. The $\gamma_2^{+/-}$ mouse spends less time in the open arms of the EPM and less time in the lit area of the light–dark box, typical of increased anxiety-type behavior. In addition, $\gamma_2^{+/-}$ mice show increased responses in the passive avoidance paradigm. This is consistent with enhanced emotional memory for negative associations, a common feature of several anxiety disorders. These behavioral alterations are associated with a lower single-channel conductance, a pronounced deficit of functional receptors, and a reduction in α_2 /gephyrin containing postsynaptic GABA $_A$ receptor clusters in cortex, hippocampus, and thalamus. Transgenic mice overexpressing either the mouse γ_{2L} or γ_{2S} subunits of the GABA $_A$ receptor showed no difference in anxiety-related behavior as compared to wild-type littermates [197]. Since compensation at the level of GABA $_A$ receptor subunit expression and assembly often occurs when subunit expression is disturbed (see below), it would be important to know the expression of all subunits in these mice. Among the α subunits, α_1 is predominant in GABA $_A$ receptors [198]. Two groups have independently generated mice with a deleted α_1 subunit and found no evidence for increased anxiety or other behavioral abnormalities [199–201]. However, an additional study demonstrated that lack of the α_1 subunit is compensated and substituted by other α subunits, presumably during development, mitigating the effect of the genetic deletion [199]. Although α_2 -subunit-deficient mice have been generated and a point mutation in this subunit (H101R) abolishes the anxiolytic effect of diazepam [202, 203], no published data exist on the behavior of these mice

except a faster habituation to a novel environment of the α_2 -subunit-deficient mice (which was interpreted as less activity in a novel environment) [204]. Therefore it is not clear if these mice have an increased anxiety phenotype. An additional complication of the interpretation of the role of the α_2 subunit can be compensation by other α subunits as occurs in the α_1 -subunit-deficient mice (see above). “Knockin” mice in the α_5 subunit (H105R) display enhanced trace fear conditioning to threat cues [196]. This is somewhat surprising because similar knockouts in the $\alpha_{1/2}$ subunits have no behavioral problems (see above). Further analysis showed that the knockin mice exhibit a 33% reduction in hippocampal (CA1 and CA3) α_5 receptor subunits; thus, these mice should be considered a partial knockout [196]. Also, α_5 receptor subunit null mutant mice exhibit improved performance in the water maze of spatial learning task but no change in locomotor activity in a novel environment [205]. Although the behavioral characterization of these mice is far from complete, it seems that the α_5 subunit is involved in hippocampal memory rather than in anxiety-related processes. Finally, the genetic inactivation of β_2 , another predominant subunit, resulted in a more than 50% reduction in the total number of GABA_A receptors and increased locomotor activity in the open field, suggesting that these receptors may control motor activity [200].

Besides the GABA_A receptor, the 5-HT_{1A} receptor has long been implicated in the pathogenesis of anxiety disorders. In 1998, three groups reported the generation of 5-HT_{1A} receptor knockout mice on different strain backgrounds [38, 206–209]. All three groups reported that the mutant mice exhibit consistently enhanced anxiety-like behaviors alongside reduced immobility in the forced-swim test [209] or tail suspension test [207, 208], indicating an antidepressant-like effect. Anxiety-related tests in these studies included open field, EPM and EZM, and novelty-induced suppression of feeding as well as fear-conditioning paradigms [207–210]. The consistency in these reports is rather remarkable because of the difference in the targeting constructs and genetic backgrounds. 5-HT_{1A} receptors are expressed both at postsynaptic locations in 5-HT target areas (such as amygdala, hippocampus, and cortex) and presynaptically on 5-HT neurons in the raphe nuclei as somatodendritic autoreceptors. Since autoreceptors control neuronal firing, it was first believed that the anxiety phenotype of the 5-HT_{1A} receptor knockout mice was the result of an increase in 5-HT release and activation of other 5-HT receptor subtypes. However, basal 5-HT levels are not altered, as measured by *in vivo* microdialysis, in 5-HT_{1A} receptor null mice [47, 211–213], and expression of 5-HT_{1A} receptors in forebrain regions rescued the phenotype of 5-HT_{1A} receptor knockout mice [214], suggesting that the behavioral phenotype results from the absence of postsynaptic 5-HT_{1A} receptors. Another interesting feature of the 5-HT_{1A} receptor knockout mice is that their anxiety-like behavior is likely the result of an irreversible early postnatal developmental abnormality [214]. In addition to increased avoidance, 5-HT_{1A} receptor knockout mice display reduced locomotor activity, another sign of increased anxiety-like behavior [214]. Another characteristic of anxiety, increased autonomic arousal (Figs. 1.2 and 1.3), was also observed in these mice. Specifically, following exposure to injection or novelty-induced stress, 5-HT_{1A} receptor knockout mice exhibited a significantly greater increase in heart rate and body temperature than wild-type mice [215, 216]. Another group reported a similar effect following footshock [37]. Taken together, 5-HT_{1A} receptor knockout mice show abnormalities in three important measures of anxiety: increased avoidance, decreased locomotor

activity, and increased autonomic arousal following exposure to a novel environment or stress. Moreover, these behavioral changes are reproducible across laboratories, which makes this genetic anxiety model not only one of the best studied but also the most robust so far in terms of the behavioral phenotype.

Another member of the 5-HT receptor family whose deletion has been associated with an alteration in anxiety levels is the 5-HT_{1B} receptor. 5-HT_{1B} receptors are predominantly localized to nerve terminals and serve as both auto- and hetero-receptors to inhibit neurotransmitter release [217, 218]. The open-field test indicated reduced anxiety-like behavior in 5-HT_{1B} receptor knockout mice [219], suggesting that this receptor may have an opposite function than that of the 5-HT_{1A} receptor (see above). Reduced anxiety was also seen in the novelty-induced suppression of feeding test [219]. However, the light–dark box and EPM tests showed no significant change in anxiety-like behavior in the 5-HT_{1B} receptor knockout mice [94, 220]. A further complication with this strain is that its behavioral phenotype was not reproducible in different laboratories even if the source of the mice was identical [220]. A similar reduced anxiety-like behavior was recently reported in 5-HT_{2A} receptor deficient mice in the EPM, open field and the light-dark box test [220a]. Importantly, the selective cortical re-expression of the 5-HT_{2A} receptor rescued the reduced anxiety-like behavior of 5-HT_{2A} receptor knockout mice indicating a role for cortical 5-HT_{2A} receptors in the modulation of conflict based anxiety-related behavior [220a].

There are two known CRH receptors (R1 and R2) and both have been suggested to be important in regulating anxiety levels. As discussed above, there are two ligands for these receptors: CRH and urocortin. Both CRH and urocortin are potent mediators of the endocrine, autonomic, behavioral, and immune responses to stress [221, 222]. CRH-R1 has a widespread distribution with high levels in anterior pituitary, hippocampus, amygdala, and cerebellum. While in the anterior pituitary CRH-R1 is involved in the activation of the HPA axis, in other regions it is responsible for the central action of CRH/urocortin and its activation is anxiogenic. In contrast to CRH-R1, expression of CRH-R2 in the CNS is restricted to the lateral septum and the ventromedial nucleus of the hypothalamus. While mice lacking CRH-R1 display decreased anxiety in the light–dark box and the EPM [223, 224], CRH-R2-deficient mice, generated independently by three groups, exhibit varying degrees of anxiety-related behavior. Bale et al. reported an increased anxiety in the EPM and open field but not in the light–dark box test in CRH-R2-deficient mice [225]. In the study of Kishimoto et al. [226], only male CRH-R2^{-/-} mice exhibited anxious behavior in the EPM and light–dark box but, paradoxically, spent more time in the center of the open field, which is more consistent with reduced anxiety. However, Coste et al. found no significant change in anxiety behavior in the EPM or open field [227]. Although not all studies are consistent with a simple interpretation, the behavioral data obtained with various CRH-R knockout mice indicate that CRH and/or urocortin mediate a dual modulation of anxiety behavior. Activation of CRH-R1 appears to be anxiogenic while activation of CRH-R2 is anxiolytic. Therefore it may not be surprising that dual CRH-R1/2 knockout mice have only a subtle behavioral phenotype; specifically, females have a reduced anxiety-like behavior in the EPM but not in the open field while males show no behavioral abnormalities related to anxiety at all [228].

As mentioned earlier, DA, presumably by regulating reward and activity, is believed to be involved in anxiety-like behavior. In particular, DA D_2 receptors have been implicated in anxiety-related behavior. Consistent with these data, DA D_3 receptor knockout mice display reduced anxiety in the open field and EPM and increased locomotor activity [229]. In contrast, D_4 knockout mice exhibit enhanced anxiety in the open-field test in the presence of a novel object [230]. While altered anxiety levels were evident, the authors interpreted much of the behavioral phenotype as changes in exploratory behavior.

Although a role for the cannabinoid 1 (CB-1) receptor is less known in anxiety-like behavior, it has been reported that CB-1 knockout mice have increased anxiety-like behavior in the light–dark box [231] and reduced exploration of the open arms of the EPM apparatus [232]. However, evidence for reduced anxiety was found in CB-1 knockout mice in the shock-probe burying test, in which anxiety is reflected by increased burying, corresponding to increased active avoidance [233].

Although the NE system and the locus ceruleus (LC) are clearly significant in the pathogenesis of anxiety as well as in animal models of anxiety, there are relatively few studies that specifically tested the role of adrenergic receptors in these conditions. So far, the α_{2a} -adrenergic receptor has been studied (among the α_{2a} , α_{2b} , and α_{2c} receptors) and mice deficient in this receptor show increased anxiety-like phenotype in various tests [234].

Consistent with the “calming” effects of adenosine and anxiety-inducing nature of caffeine, rats treated with a nonspecific antagonist at adenosine receptors [235] as well as adenosine_{2a} (A_{2a}) receptor null mice exhibit increased avoidance in the EPM and light–dark box, decreased exploratory behavior, and decreased locomotor activity (reduced activity), typical signs of increased emotionality and anxiety [236] (see also Fig. 1.3). The A_{2a} receptor is co expressed with DA D_2 receptors in GABAergic neurons in basal ganglia and striatum and is thought to regulate the expression of the proenkephalin gene [237]. In situ hybridization studies showed a decrease in proenkephalin gene expression in the A_{2a} receptor knockout mice, which may explain the anxiety-like behavior of these mice.

G-protein-gated inwardly rectifying K^+ (GIRK) channels contribute to post-synaptic inhibition triggered by many neurotransmitters, including DA and 5-HT, and GIRK2-deficient mice have been found to display a phenotype consistent with reduced anxiety [238]. Four GIRK subunits (GIRK1 to GIRK4) have been identified, and tetrameric channels formed by various combinations of GIRK1, GIRK2, and GIRK3 mediate inhibition in the nervous system [239, 240]. In addition to less avoidance in the EPM and light–dark box test, GIRK2 knockout mice also display increased locomotor activity satisfying two criteria of reduced emotionality (see Fig. 1.3).

Nicotinic agonists and antagonists can modulate anxiety [241, 242], and mice with a null mutation in the nicotinic acetylcholine receptor (nAChR) α_7 subunit gene have been shown to exhibit decreased anxiety in the open field but not in the light–dark box [243]. In contrast, mice deficient in the nAChR α_4 -subunit gene display increased anxiety in the EPM [244], indicating that the subunit composition of the nAChR may determine whether the effect is anxiogenic or anxiolytic.

One of the targets of BDNF is trkB, a receptor tyrosine kinase [245]. Consistent with the increased anxiety-like phenotype of the conditional BDNF mutant mice

[246], transgenic mice overexpressing *trkB* in postmitotic neurons in a pattern similar to that of the endogenous receptor display less anxiety in the EPM test [246].

Neurotransmitter and neuromodulator receptors are not the only substrates of communicating external signals into neurons. Cell–cell interactions are crucial in regulating neuronal functions and developmental processes. One group of proteins that mediate cell–cell interactions is represented by neuronal adhesion molecules that regulate, among others, synaptic plasticity in both the developing and adult brain. Recent studies indicate that neuronal cell adhesion molecules of the immunoglobulin superfamily (NCAM and L1) are important mediators of the effects of stress. Chronic stress alters the expression pattern of cell adhesion molecules in parallel with their effects on behavior [247]. The connection between neuronal cell adhesion molecules and emotional behavior is also supported by the change in the anxiety-like phenotype of NCAM and L1 null mice. Genetic inactivation of NCAM results in decreased anxiety in the light–dark and EPM tests [248]. In addition, these mice respond to the anxiolytic effect of buspirone in the light–dark test at lower doses than the NCAM^{+/+} mice, suggesting that there may be an alteration in the sensitivity of the 5-HT_{1A} receptors in these knockout mice. However, the authors reported no changes in the density of 5-HT_{1A} receptors or in tissue 5-HT content. Since NCAM has been demonstrated to have a role in CNS development and neuroplasticity, a developmental abnormality may explain the expression of anxiety-like behavior in these mice (similarly to the BDNF, IFN γ , and 5-HT_{1A} knockout mice; see Sections 1.9 and 1.10). Also, conditional inactivation of L1 in the forebrain, mostly from early postnatal life [by cre-recombinase under the control of the calcium/calmodulin-dependent protein kinase II (CaMK II) promoter], resulted in decreased anxiety in the open field and EPM [249]. Conditional expression avoids the severe morphological and behavioral abnormalities associated with the absence of L1 during prenatal development. Finally, the lack of cadherin-11, another cell adhesion molecule, results in reduced fear- or anxiety-related responses [250]. Cadherin-11 is expressed in the limbic system of the brain, most strongly in the hippocampus, and is densely distributed in synaptic neuropil zones. Taken together, the loss of function of three cell adhesion molecules leads to maladaptive behavioral responses that are “opposite” to anxiety and may be characterized as excessive novelty seeking and a lack of appropriate response to danger (see Fig. 1.3, blue region of the cube, and discussion in the accompanying text). It is striking that all three adhesion molecules mentioned above are involved in the regulation of synaptic structure and function [251]. This indicates that the optimal functioning of synapses is essential for mediating appropriate responses to novelty and stress.

1.11 INTRACELLULAR REGULATORS ASSOCIATED WITH ANXIETY-LIKE PHENOTYPE

A number of intracellular signaling molecules and transcription factors have been shown to cause increased or reduced anxiety-like phenotype in mice. The α isoform of CaMKII is an important second messenger, and Chen et al. [252] demonstrated decreased anxiety in CaMKII knockout mice. CaMKII is a major component of the postsynaptic density in glutamatergic synapses [253] and is involved in neuronal

functions related to calcium signaling, including the induction of long-term potentiation (LTP) [254]. Therefore, the disruption of CaMKII function could alter many aspects of neuronal function, making it difficult to relate it to a specific anxiety behavior. Indeed these knockout mice also exhibit enhanced aggression and learning impairment.

The serine/threonine kinase protein kinase C γ (PKC γ) has recently been shown to be a regulator of anxiety behaviors [255]. PKC γ is restricted to the CNS and is highly expressed in limbic areas of the brain. In three different behavioral tests (EPM, light–dark test, mirrored chamber) PKC γ knockout mice consistently showed reduced anxiety-like behavior. Bowers et al. [255] proposed that PKC γ modulates anxiety by altering the function of GABA_A, *N*-methyl-D-aspartate (NMDA), or 5-HT₂ receptors.

Another intracellular signaling molecule implicated in anxiety and fear responses is the tyrosine kinase Fyn. Fyn is a member of the Src family of tyrosine kinases that can associate with and phosphorylate a variety of molecules. Inactivation of the *fyn* gene in mice results in increased anxiety-like behavior to naturally aversive stimuli in the light–dark box and novelty tests [256]. These mice also display enhanced learned fear responses in the passive-avoidance test. Fyn is highly expressed in the limbic system and has been implicated in NMDA receptor-mediated synaptic plasticity, NCAM-dependent neurite outgrowth, and myelination [257–259]. Whether any or all of these processes are involved in the enhanced anxiety exhibited in the Fyn^{−/−} mice is unclear.

The NF- κ B transcription factor family is linked to a number of receptors, including TNF α , and controls the expression of many genes involved in cell survival, proliferation, and regulation of inflammatory and stress responses. It has recently been shown that mice lacking the p50 subunit of NF- κ B have a reduced anxiety-like phenotype [260]. These mutant mice showed reduced avoidance and autonomic arousal in the open field and EPM. In immune cells, NF- κ B factors are kept inactive by association with inhibitory proteins belonging to the I κ B family and activating stimuli induce the phosphorylation, polyubiquitination, and proteasome degradation of I κ Bs, allowing NF- κ B to translocate into the nucleus and activate target genes. In contrast, it seems that either NF- κ B is constitutively active in neurons [261] or normal neuronal activity is sufficient to keep a substantial amount of NF- κ B in an active form. Since it is expressed during development [262], NF- κ B may regulate the development of brain circuits, and consequently the reduced anxiety-like phenotype of p50 knockout mice could be due to abnormal brain development.

GR is another transcription factor (activated via the HPA axis and glucocorticoid hormones) and is well known to be involved in stress response and some anxiety disorders. As discussed later, a brief period of controllable stress experienced with general arousal and excitement can be beneficial, but chronically elevated levels of circulating corticosteroids are believed to enhance vulnerability to a variety of diseases, including affective disorders. Therefore it is not surprising that the genetic manipulation of GR results in changes in emotionality in mice. Reduced anxiety-like phenotype was found in a brain-specific GR knockout [263] and in a GR-antisense model with reduced GR expression in brain and some peripheral tissues [264], while GR overexpression in forebrain results in increased anxiety-like behavior [265]. Together, these findings indicate that a sustained increase in GR activity in brain is associated with increased anxiety-like behavior.

In addition to its role in the regulation of calcium and phosphate homeostasis and in bone formation, vitamin D is also thought to be involved in brain function. Genetic ablation of the vitamin D receptor (VDR), another nuclear receptor linked to transcription, results in increased anxiety-like behavior in a battery of behavioral tests [266].

Still another group of transcription factors associated with anxiety-like behavior is the family of cyclic adenosine monophosphate (cAMP)-responsive nuclear factors that consist of CREB, CRE modulator (CREM), and activating transcription factor 1 (ATF-1) [267]. A conditional CREB mutation that inactivates all isoforms in the brain or the disruption of the two major transcriptionally active CREB isoforms (α and δ) increases anxiety-like responses in mice in different behavioral tests, including the EPM [268]. In CREB-deficient mice, the expression of CREM isoforms is increased [269]; thus, the higher anxiety-like phenotype may be attributable to this change. Indeed, CREM-deficient mice display reduced anxiety-like behavior in the EPM test and also exhibit hyperactivity [270], indicating that CREM activity may be linked to neuronal modulation promoting anxiety.

BC1 RNA is a small non-messenger RNA common in dendritic microdomains of neurons in rodents, and it is believed to play a role in translational regulation. Mice mutant for BC1 show behavioral changes consistent with increased anxiety and reduced exploration [271]. These data indicate that an anxiety-like phenotype can be induced by disturbing gene expression beyond transcription at the translational level.

Taken together, defects in intracellular processes involving second messengers, transcription factors, and translational factors can lead to alterations in anxiety in mice. To better understand the neurobiology of anxiety, it will be critical to identify the specific mRNAs and proteins whose altered synthesis in neurons of the fear/anxiety pathway is associated with the expression of the behavioral phenotype. In any event, the common feature of these molecular changes is that they could all eventually influence morphological and/or functional plasticity in the nervous system (see further discussion on this topic below).

1.11.1 Modeling Complex Genetics of Anxiety in Mice: Oligogenic Anxiety-Like Conditions in Mice

Genetic studies on various mouse phenotypes clearly indicate that most behavioral traits are heritable and are specified by multiple genes or QTLs. For example, mapping studies have estimated that the individual anxiety-related behavioral differences in the DeFries recombinant inbred strains of mice are the result of the interaction between four to six QTLs for each behavior; the largest QTL explains no more than half of the variance attributable to the detected QTL [15, 272, 273]. However, QTLs can consist of hundreds of genes, and these studies are not designed to analyze the contribution of individual genes to anxiety. An alternative strategy to study the combined effect of two or more genes on behavior is to use double- and triple-knockout mice. A recent report analyzed anxiety-related behaviors in double 5-HTT^{-/-} and BDNF^{+/-} mutant mice [274]. These mice, as compared to 5-HTT^{-/-}, BDNF^{+/-}, and wild-type mice, displayed a significantly higher level of anxiety-like behavior, reduced levels of 5-HT and 5-hydroxyindole acetic acid in the hippocampus and hypothalamus, and greater increases in plasma ACTH after a

stressful stimulus. These findings support the hypothesis that genetic changes in BDNF expression interact with 5-HT to modulate anxiety and stress-related behaviors.

Another double-knockout strain lacking both monoamine oxidase (MAO) A and B, two enzymes responsible for the degradation of monoamines, shows anxiety-like behavior in various tests [275]. Neither the MAO_A nor the MAO_B knockout mutants display anxiety in these tests, indicating that an interaction between the two MAO genes leads to a novel phenotype [276, 277]. Since monoamine levels are higher in the double knockouts than in the single mutants, the abnormal behavior of MAO_{A/B} mutants is likely the consequence of altered monoaminergic neurotransmission.

1.12 EFFECTS OF EARLY-LIFE ENVIRONMENT ON ANXIETY

1.12.1 Early-Life Experience on Expression of Anxiety in Later Life

A large body of evidence supports the notion that early-life environmental effects alter life-long stress-coping mechanisms. Unlike human studies, which are predominantly retrospective with a large number of environmental variables, animal research has focused on the effect of “handling” and maternal care during postnatal development. Brief handling of pups results in life-long decreases in behavioral and endocrine responses to stress while animals separated from their mothers/litters for longer periods of time (i.e., for several hours) exhibit increased anxiety [278–280]. Later studies determined that the critical feature of short-term handling was the increase in maternal care [licking and grooming (LG)] following the return of pups [281]. Variability in maternal care can produce large differences in adult behavior and hormonal responsiveness to stress. Pups nursed by mothers with either a high or a low level of LG and arched-back nursing (ABN) show a decreased and increased level of anxiety-like behavior in adult life (open field, novelty-suppressed feeding, and shock-probe burying assays), respectively [281–284]. Also, offspring of high LG–ABN mothers (as well as briefly handled pups) have reduced plasma levels of ACTH and corticosterone in adulthood following stressful stimuli such as restraint stress when compared to the offspring of low LG–ABN mothers (or nonhandled pups) [285]. Furthermore, these animals show increased glucocorticoid feedback sensitivity, increased hippocampal GR mRNA expression, and decreased hypothalamic CRH mRNA levels.

Interestingly, pups born to low LG–ABN mothers but cross fostered to high LG–ABN mothers develop low anxiety-like behaviors in adulthood, but high LG–ABN pups reared by low LG–ABN mothers do not develop increased anxiety-like responses in adulthood [285]. Furthermore, the maternal behavior of female offspring from low LG–ABN mothers can be changed by cross fostering them to high LG–ABN mothers. The reverse, however, is not true because daughters of high LG–ABN mothers raised by low LG–ABN dams have high LG–ABN maternal behavior [281, 286]. Finally, offspring of low LG–ABN mothers, if cross fostered to high LG–ABN mothers, show hormonal levels similar to those observed for offspring of high LG–ABN mothers [285]. These findings suggest that environmental effects may overpower genetic predispositions, particularly in cases where such modification would be beneficial for survival.

Although experiments related to both postnatal handling and maternal behavior clearly show a nongenomic influence on anxiety-like behavior, the transmission

mechanism of this effect has been difficult to elucidate. It has been hypothesized that environmental influences exert some level of control on the development of HPA and the regulation of HPA function via a number of neurotransmitter systems, including the noradrenergic, GABAergic, and glutamatergic systems [287, 288]. For example, rat pups of high LG-ABN dams show altered GABA_A receptor subunit expression in the amygdala, LC, medial prefrontal cortex, and hippocampus that could contribute to their reduced anxiety-like behavior as compared to pups from low LG-ABN dams [289, 290]. In addition to the GABAergic system, other potential factors mediating the environmental effects include the glutamatergic system and neurotrophins such as BDNF. Liu et al. [291] found that adult offspring of high LG-ABN Long Evans dams show increased hippocampal synaptogenesis and better spatial learning and memory than low LG-ABN offspring and that these differences could be equalized if low LG-ABN pups were cross fostered to high LG-ABN dams. More specifically, increased LG-ABN of offspring resulted in increased hippocampal mRNA expression of NR2A and NR2B NMDA receptor subunits at postnatal day 8, a change that was sustained into adulthood. Consistent with the regulation of the BDNF gene by NMDA receptors [292], increased levels of BDNF, but not NGF or NT-3, mRNA were observed in the dorsal hippocampus of eight-day-old high LG-ABN pups [291]. Most recently, it has been reported that the maternal effect is linked to alterations in methylation and chromatin structure at the GR promoter in the offspring [291a]. It has been proposed that downregulation of hippocampal GR in the pups of low nursing mothers compromises feedback inhibition in the hypothalamic pituitary adrenal axis ultimately leading to higher anxiety states [291a]. Since GR knockout mice have reduced anxiety [263] and GR overexpression in forebrain results in increased anxiety-like behavior [265], it is possible that the maternally-induced regulation is specific for a subset of GRs and does not involve the GR pool implicated in the stress related actions of glucocorticoids.

1.12.2 Interaction of Environment with Genes in Establishing Level of Anxiety

Although the interaction of genes and environment in shaping personality is well accepted, direct experimental evidence to support this notion has been difficult to obtain in humans. Recent association studies, however, have clearly indicated that genetic and environmental factors act together, enhancing the phenotype beyond the level established by either factor alone. For example, promoter polymorphism in 5-HTT can influence anxiety-related behavior (*s/s* genotype represents a predisposition to neuroticism/anxiety) [9], and a recent report showed that this polymorphism moderates the influence of stressful life events on depression. Individuals with one or two copies of the *s* allele of the 5-HTT promoter polymorphism exhibited more depressive symptoms in relation to stressful life events than *l/s* individuals [293]. Although this study was focused on depression, anxiety is a common symptom in depression, and future studies may reveal evidence of an interaction between an individual's 5-HTT allelic makeup and environmental insults in anxiety disorders. This connection has already been made in primates. Rhesus monkeys have a 5-HTT polymorphism similar to that found in humans, and it was shown that although both mother-reared and nursery-reared heterozygote (*l/s*) animals demonstrate increased affective responding (a measure of temperament) relative to *l/l* homozygotes,

nursery-reared but not mother-reared *l/s* infants exhibited lower orientation scores than their *l/l* counterparts [294]. Also, monkeys with deleterious early rearing experiences were differentiated by genotype in cerebrospinal fluid concentrations of the 5-HT metabolite, 5-hydroxyindoleacetic acid, while monkeys reared normally were not [295]. Another study found that separation-induced increases in ACTH levels were modulated by both rearing condition and 5-HTT polymorphism [296]. During separation, animals with *l/s* genotypes had higher ACTH levels than *l/l* animals, and peer-reared *l/s* animals had higher ACTH levels than all other groups, including mother-reared animals.

Rodents are more amenable to such studies, and there have been a number of reports on the effect of early-life experience and gene interaction on later-life behavior. For example, early-life handling or cross fostering of highly neophobic BALB/c mice to less neophobic C57BL/6 mice equalizes both the behavioral and the benzodiazepine receptor expression differences between these two strains as well as decreasing the ACTH release following an acute stressor of BALB/c mice in adulthood [284, 297–299]. Furthermore, the effect of the 5HT_{1A} receptor gene on the anxiety-like behavior may be modulated by the environment. In a recent study, Weller et al. documented that F1 5HT_{1A}R^{+/-} offspring reared by 5HT_{1A}R^{-/-} mothers have increased ultrasonic vocalization (USV) when compared to F1 5HT_{1A}R^{+/-} offspring raised by 5HT_{1A}R^{+/+} dams [300]. However, contrary to expectations, F1 5HT_{1A}R^{+/-} offspring reared by 5HT_{1A}R^{-/-} mothers have decreased measures of anxiety in the EPM as adults when compared to F1 5HT_{1A}R^{+/-} offspring raised by 5HT_{1A}R^{+/+} dams. Also, 5HT_{1A}R^{-/-} pups reared by either 5HT_{1A}R^{-/-} or 5HT_{1A}R^{+/-} dams produced less isolation-induced response (USV) than their 5HT_{1A}R^{+/+} controls [300]. Although it is difficult to consolidate these seemingly contradictory results, these experiments show that the level of anxiety associated with 5HT_{1A}R deficiency can be altered by environmental factors.

In addition to early environmental influences, later-life or adult environment can also influence the expression of emotionality, as demonstrated in mouse models. As discussed previously, lack of the nociceptin/orphanin FQ gene leads to an enhanced anxiety phenotype in mice [184, 185]. The strength of the behavioral expression of the phenotype is dependent, however, on environmental influences such as social interactions. Ouagazzal et al. found that homozygous mutant animals, when housed alone, performed similarly to their wild-type controls on tests of emotional reactivity. Enhanced emotionality became apparent only when the singly housed animals were introduced to group housing (five animals per cage) that induced greater levels of aggression and increased anxiety responses [301].

Taken together, these data show that both genetic and environmental factors have an important role in establishing emotionality in mammals. Often, these factors work together, enhancing the phenotype beyond the level established by either factor alone. Other times, the environmental influences can partially or fully rescue undesirable phenotypes caused by genetic predispositions or mutations, enhancing the likelihood of the organism's survival.

1.13 CONCLUSIONS: NEUROBIOLOGY OF ANXIETY DISORDERS

Combining what is known about anxiety disorders (including symptomatology, pharmacology, and biochemistry) with the genetic and molecular information

gathered from the diverse knockout mouse strains that exhibit alterations in anxiety-like behavior, it becomes apparent that anxiety-related pathways and processes involve communication between neurons (including neuronal messengers and their receptors) and/or signaling within cells (Fig. 1.6 [302]). Since the manipulation of a ligand, its receptor, and a coupled intracellular signaling elicits a similar anxiety-like behavior, it is possible to cluster these molecules to pathways. Many of these pathways eventually converge onto regulation of transcription and/or translation, and one can hypothesize that anxiety-like behavior is the result of changes, at least in part, at the level of gene expression (Fig. 1.6). Indeed, the genetic manipulation of transcription (by CREB, GR, VDR, and NF- κ B; see previous sections) can also result in changes in anxiety levels.

Several of these “anxiety-related pathways” can be established. For example, the “serotonergic” pathway consists of receptors controlling the release of 5-HT (5-HT_{1A} and 5-HT_{1B} receptors), 5-HTT, postsynaptic 5-HT receptors, and mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK) signaling (Fig. 1.6). As described in this chapter, a change in any of these components can lead to altered emotional behavior. The proper function of this pathway is especially crucial during early postnatal development, and one can hypothesize that abnormal signaling via this system alters the development of neuronal networks and consequently function, manifested as abnormal fear/anxiety response. BDNF, whose deficiency has also been associated with anxiety-like behavior, is also linked to the MAPK–ERK signaling (Fig. 1.6), suggesting that the two distinct anxiety-associated traits (deficit in 5-HT_{1A} and BDNF) may share downstream targets. In addition to the convergence of various pathways, the same extracellular signal can diverge to various intracellular pathways illustrated by the coupling of the 5-HT_{1A} receptor to both the MAPK–ERK and NF- κ B pathways [303–307] (Fig. 1.6). Such divergence obviously broadens the clusters of affected genes. Since manipulation of the p50 subunit of NF- κ B (activated by cytokines) can also be linked to anxiety-like behavior, crosstalk is extensive at the signaling level and therefore within and between anxiety-related pathways. Although the function of 5-HT and BDNF pathways is altered not only as a result of mutations and genetic polymorphisms but also by the environment, another “anxiety-related” pathway consisting of CRH, ACTH, and GR is especially sensitive to environmental changes. As with the other pathways, activation of this pathway by chronic stress eventually alters gene regulation (via GR).

It is hypothesized that abnormalities in these pathways at any level lead to, via altered gene expression, changes in neuronal morphology and/or function. Indeed, a number of knockout mouse strains with an anxiety-like phenotype as well as rodents following chronic stress show altered dendritic arborization in hippocampus and amygdala [308, 309], abnormal synapse formation [126, 250, 310], and altered electrical properties of neurons [311]. These changes result in abnormal neuronal network activity characterized by a deficit in short-term plasticity (i.e., hippocampal paired pulse facilitation and inhibition) [210, 312, 313], abnormalities in long-term potentiation [250, 312], an increase in network excitability [210, 311, 314], and abnormal activation or inhibition of brain regions as measured by fMRI [74, 75, 78]. In anxiety disorders, multiple molecular pathways may be simultaneously affected in multiple brain regions, consistent with the multitude of associated symptoms. Importantly, all commonly used anxiolytic drugs [benzodiazepines, selective

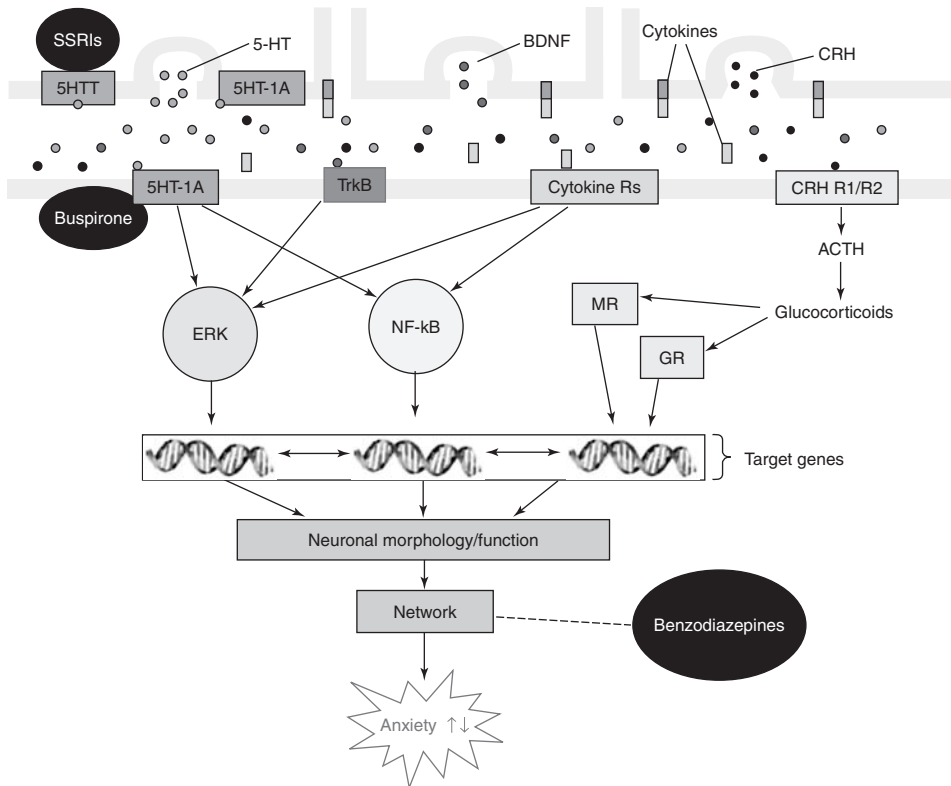


Figure 1.6 Unifying model of pathogenesis of anxiety. Pharmacological and genetic studies indicate that defects in specific substrates of neuronal communication (e.g., 5-HT, GABA, 5-HT receptors), intracellular signaling (e.g., ERK), and gene expression (GR) may be involved in anxiety-related behavior. These extracellular neuronal messengers and their associated receptors and coupled intracellular signaling form specific pathways. Abnormalities in these molecular pathways at various levels can directly modify the function/morphology of the neuronal network associated with fear, ultimately producing changes in anxiety levels. (See color insert.)

serotonin reuptake inhibitors (SSRIs), and buspirone] can be integrated into the model described above, indicating that the genetic data are consistent with the pharmacological data and that anxiolytic drugs target and modulate the molecular and cellular pathways which apparently control or establish (during development) the level of anxiety (Fig. 1.6).

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2

PHARMACOTHERAPY OF ANXIETY

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2.1	Introduction	60
2.2	Clinical Management of Anxiety	60
2.3	Diagnosis of Anxiety Disorders	61
2.4	Anxiolytic Drugs	62
2.4.1	Drugs Acting via Monoamine Neurotransmission	63
2.4.1.1	Antidepressants	64
2.4.1.2	5-HT _{1A} Receptor Agonists	70
2.4.1.3	β Blockers	71
2.4.1.4	Antipsychotics	71
2.4.2	Drugs Acting via Amino Acid Neurotransmission	72
2.4.2.1	Benzodiazepines	73
2.4.2.2	Anticonvulsants	74
2.4.3	Drugs with Other Mechanisms of Action	75
2.4.3.1	Antihistamines	75
2.4.3.2	Lithium	75
2.5	Pharmacotherapy for Anxiety Disorders	75
2.5.1	Generalized Anxiety Disorder	77
2.5.2	Obsessive-Compulsive Disorder	77
2.5.3	Panic Disorder and Agoraphobia	78
2.5.4	Post traumatic Stress Disorder	79
2.5.5	Social Anxiety Disorder (Social Phobia)	79
2.5.6	Specific Phobia	80
2.5.7	Anxiety Symptoms in Depressive Disorders	80
2.6	Conclusions and Future Directions	81
	References	82

2.1 INTRODUCTION

The medical treatment of anxiety disorders is passing through an interesting phase. The range of therapeutic options is expanding and public interest in the field is high. Longstanding controversies, such as the relative merits of psychological therapies versus medication and the safety of medical treatments of anxiety, are debated in the national media. Patients are increasingly active participants in the therapeutic process using widely available sources of medical information, while government guidelines seek to standardize prescribing practice and have provoked debate within the medical profession. The management of anxiety continues to sit uncomfortably between primary and secondary care and in many ways remains the poor relation within mental health care in most if not all countries. Nevertheless current medical practice is based on a substantial volume of research evidence and clinical experience, and it is possible to justify with confidence the treatment options put before patients.

The diagnostic concept of anxiety has undergone progressive evolution since Donald Klein described the discrimination of panic disorder from other neuroses [1]. New diagnostic categories have emerged, and although some discrepancies remain between different classifications, general agreement has been reached for the diagnostic validity of the main categories. This has allowed quantitative measurement of the prevalence and economic burden of anxiety disorders in epidemiological surveys [2, 3], and this evidence has been a key factor driving research into anxiolytic therapies.

The rapid advances of the past 15 years have also been driven by developments in a diversity of other disciplines, including preclinical and clinical pharmacology, cognitive and experimental psychology, and neuroscience (particularly neuroimaging). Research from areas such as genetics and molecular biology is only just beginning to have an impact. This chapter describes the current basis of pharmacotherapy for anxiety disorders, but with the pace of scientific discovery this is a field that is likely to undergo further change in the years ahead.

2.2 CLINICAL MANAGEMENT OF ANXIETY

Management of an anxious patient involves far more than the prescription of medication (Fig. 2.1). Establishing the diagnosis of a specific anxiety disorder is a critical part of the assessment process that may greatly influence the treatment offered. Anxiety disorders frequently present with comorbid conditions such as depression, alcohol or substance use problems, and other anxiety disorders, which must be detected and managed appropriately. A patient tends to present when anxiety causes impairment of their social, occupational, or domestic functioning, and identification of the key complaints helps in drawing up an effective management plan; for example, a patient with generalized anxiety disorder may complain that insomnia impairs his or her ability to work, and management should include strategies to improve sleep efficiency as well as treatment of the anxiety. Most anxiety disorders and any concomitant depression are associated with an increased risk of suicidal thoughts and acts of deliberate self-harm, and these should be carefully assessed and monitored during treatment.

Psychoeducation helps engagement and improves recovery. Models of anxiety disorders have been described in both the biological and psychological dimensions,

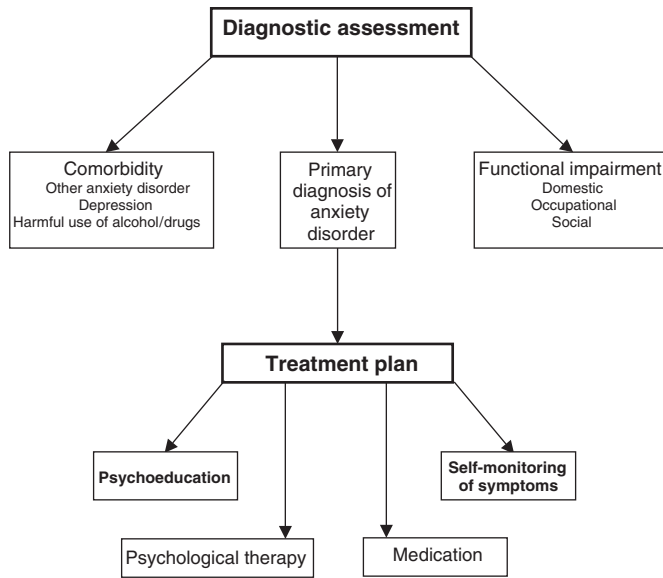


Figure 2.1 Clinical assessment of anxious patient.

and patients benefit from an explanation, tailored to their level of understanding, in each dimension. This can be reinforced by the use of educative literature [4]. Treatment efficacy is improved when patients monitor and record their symptoms [5].

A management plan is negotiated with the patient by considering any appropriate biological and psychological treatments. A combination of drug and psychological therapies can be more effective than either alone [6]. The patient may have preconceptions about specific therapies, often as a result of their anxiety; for example, a patient with panic disorder fears the effects of drugs and a patient with social anxiety baulks at the suggestion of group therapy. An open discussion of benefits and adverse effects, including long-term side effects, is likely to improve compliance. Although medications are generally well tolerated, some side effects commonly occur, and anxious patients tend to experience more than others [7]. Progress with treatment should be encouraged by regular review, particularly in the early stages.

2.3 DIAGNOSIS OF ANXIETY DISORDERS

Although every method for categorizing anxiety disorders has its shortcomings, in current clinical practice the diagnostic criteria of the American Psychiatric Association [fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)] is most commonly used [8]. *Anxiety disorder* is divided into syndromes with clear operational criteria. In particular, the criteria are clearly stated for a symptomatic individual to become a case, and this diagnostic threshold is defined either by the level of distress experienced by the individual or in terms of impairment of occupational, social, or domestic functioning. Patients may have symptoms from more than one diagnostic category, but it is important to elicit the primary diagnosis, as this will influence the recommended treatment. Comorbid disorders, usually a

TABLE 2.1 Diagnostic Criteria for Major Anxiety Disorders

Generalized anxiety disorder (GAD)	Excessive worry/anxiety about various matters for at least 6 months; difficulty in controlling worry; accompanying somatic symptoms (effects of chronic tension); clinically important distress or impairment of functioning
Obsessive-compulsive disorder (OCD)	Presence of obsessions (thoughts) or compulsions (behaviors); symptoms felt by patient to be unreasonable or excessive; clinically important distress or impairment of functioning
Panic disorder with/without agoraphobia ^a	Severe fear or discomfort peaking within 10 min; characteristic physical/psychological symptoms; episodes are recurrent and some unexpected; anxiety about further attacks or consequences of attacks
Post-traumatic stress disorder (PTSD)	Severe traumatic event that threatened death or serious harm; felt intense fear, horror, or helplessness; repeated reliving experiences; phobic avoidance of trauma-related stimuli; hyperarousal; symptoms lasting > 1 month and causing clinically important distress or impairment of functioning
Social anxiety disorder	Recurrent fears of social or performance situations; situations avoided or endured with distress; clinically important distress or impairment of functioning
Specific phobia	Persistent fear/avoidance of specific object or situation; phobic stimulus immediately provoking anxiety response; clinically important distress or impairment of functioning.

Source: After DSM-IV.

^aAgoraphobia: anxiety about place/situation where panic attack is distressing or escape difficult; situation is avoided, endured with distress or companion is required.

second anxiety disorder, mood disorder, or substance use disorder, are common and must be detected. The key diagnostic criteria for the major anxiety disorders are given in Table 2.1.

2.4 ANXIOLYTIC DRUGS

The use of substances for their anxiolytic properties dates from the beginning of recorded human history. The last century saw the development of their use for medical purposes, and major progress was made in the 1990s, “the decade of anxiety,” as advances in neuroscience provided a basis for the design of new treatments. There are now drugs available that are better tolerated, although not necessarily more effective, than their predecessors. Despite growing knowledge of the complex neural pathways involved in anxiety, it is notable that the actions of current drugs occur via a small number of neurotransmitter systems, with the most important being the monoaminergic neurotransmitters [serotonin (5-HT), noradrenaline, and to a lesser extent dopamine] and the amino acid neurotransmitters [chiefly γ -aminobutyric acid (GABA) but also glutamate].

2.4.1 Drugs Acting via Monoamine Neurotransmission

Interest in this field originated with the serendipitous discovery of the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), when these drugs were later found to exert their anxiolytic effects via actions on monoamine systems. Recent research, rather than clarifying the role of these neurotransmitters in anxiety pathways, has tended to present an increasingly complex picture [9]. Nevertheless these advances have led to the development of “designer drugs” with selective effects on serotonergic and noradrenergic neurones. Drugs such as the selective serotonin reuptake inhibitors (SSRIs) do not exceed their predecessors in terms of anxiolytic efficacy [10], but improved tolerability has led to their adoption as first-line treatments for anxiety disorders. The historical development of drugs in this field is illustrated in Fig. 2.2.

The monoamines are relatively simple molecules that are synthesized within neurons from dietary amino acids. They facilitate transmission in neural pathways that originate in brain stem nuclei and have descending projections to the autonomic nervous system and widespread ascending projections to sites in the limbic system and cortex. In addition to the control of anxiety responses, these pathways modulate many other aspects of behavioral function, including mood, appetite, and sleep.

Among the monoamines the role of 5-HT in anxiety is best understood, but the picture is complex as increased serotonergic activity may be anxiogenic or anxiolytic depending on the site and duration of action [11]. 5-HT is released into the synapse and either binds to postsynaptic receptors or reenters the neuron via a specific transporter (Fig. 2.3). The serotonergic neuron regulates its own firing rate via the action of inhibitory autoreceptors. Anxiety disorders have been shown to be associated with various alterations in the serotonergic neuron including reduced

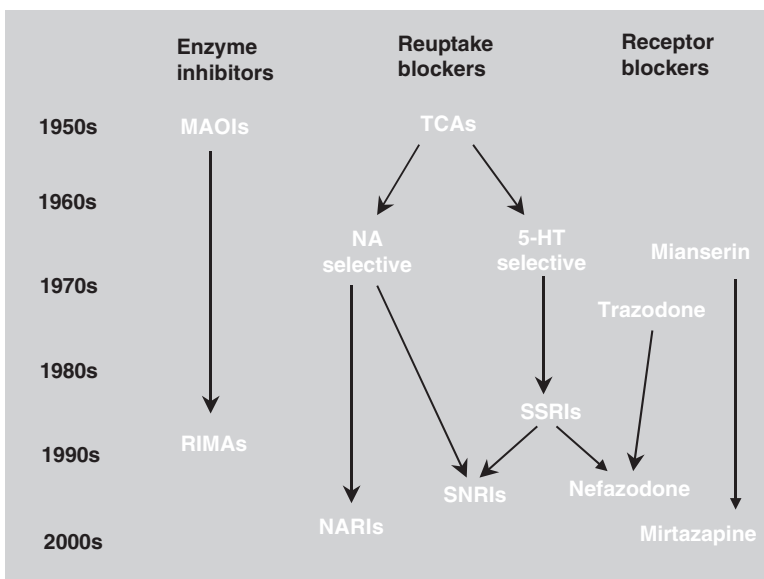


Figure 2.2 Historical development of antidepressant drugs.

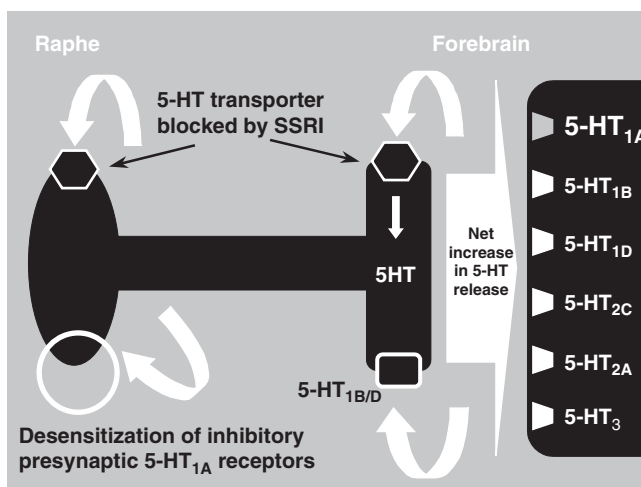


Figure 2.3 Actions of anxiolytic drugs on serotonergic neurone.

binding to presynaptic and postsynaptic 5-HT_{1A} receptors [12] and possibly with a genetic variation in the 5-HT transporter [13].

Anxiolytic drugs can alter monoaminergic neurotransmission by increasing synaptic availability or by direct action on postsynaptic receptors. Mechanisms for increasing monoamine availability include promoting release by blocking inhibitory autoreceptors, decreasing reuptake by blocking transporters, and decreasing metabolism by inhibiting oxidative enzymes. Monoamines are also implicated in the pathophysiology of depression, and drugs that increase their synaptic availability tend to have antidepressant effects. These drugs have been traditionally classified as antidepressants, although they have a primary role as anxiolytics and are currently the major drug class in the field. Other anxiolytic drugs acting via monoamines are the postsynaptic serotonin receptor partial agonist buspirone, the β -adrenoceptor blockers, and drugs classed as antipsychotics, which are primarily antagonists at postsynaptic monoamine receptors.

2.4.1.1 Antidepressants. Taken together the efficacy of antidepressants covers the spectrum of anxiety disorders, although there are important differences between drugs in the group (Table 2.2). Several new antidepressants have been marketed since the SSRIs: venlafaxine and mirtazapine are discussed later; nefazodone, a serotonin reuptake inhibitor and postsynaptic 5-HT₂ blocker, showed promise in early studies but was recently withdrawn by its manufacturers; and reboxetine, a noradrenaline reuptake inhibitor (NARI), showed benefits in panic disorder in one published study [14] and further evidence of its anxiolytic efficacy is awaited.

There are important differences between antidepressants and other drug groups in the onset and course of their actions (Fig. 2.4). There is often an increase in anxiety on initiation of therapy, and anxiolytic effects occur later. In comparative studies improvement matches that on benzodiazepines after four weeks [15]. Withdrawal effects, particularly rebound anxiety, are less problematic with antidepressants than with benzodiazepines, although stopping treatment is associated with a significant

TABLE 2.2 The Use of Antidepressants in Anxiety Disorders

Parameter	SSRI/SNRI ^a	TCA	Mirtazapine	MAOI/RIMA ^b
Efficacy	GAD, OCD, panic disorder PTSD, social anxiety disorder	Panic disorder, OCD (GAD, PTSD)	Panic disorder, PTSD	Panic disorder, PTSD, social anxiety disorder
Tolerability	Onset worsening, side effects on initiation, few long-term effects	Onset worsening, side effects on initiation, some long-term effects	Few side effects on initiation, few long- term side effects	Significant short- and long- term side effects, special dietary requirements, moclobemide better tolerated
Safety	Relatively safe in overdose	Significant overdose toxicity	Relatively safe in overdose	Significant overdose toxicity (less with moclobemide)
Discontinuation syndrome	Well-described, more common with paroxetine and venlafaxine, uncommon with fluoxetine	Well described	Not reported	Reported

Note: For items in brackets the evidence is less strong.

^aSerotonin and noradrenaline receptor inhibitor.

^bMonoamine oxidase inhibitor/reversible inhibitor of monoamine oxidase.

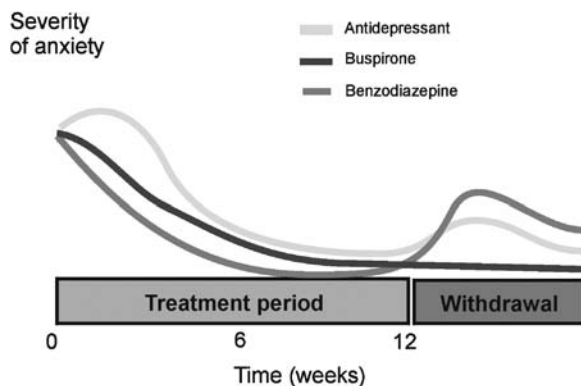


Figure 2.4 Onset and course of action of anxiolytic drugs. (See color insert.)

risk of relapse and a withdrawal (discontinuation) syndrome has been described for most of the antidepressants with short half-lives.

2.4.1.1.1 SSRIs. These drugs increase the availability of synaptic 5-HT by selectively blocking the 5-HT reuptake transporter (Fig. 2.3). In preclinical and human studies acute doses are anxiogenic [16] but chronic administration has anxiolytic effects, possibly due to downregulation of presynaptic autoreceptors [17]. There are five SSRIs widely available: citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline. Escitalopram, the S enantiomer of citalopram, has been demonstrated to be effective in several studies of anxiety disorders and has the same spectrum of efficacy as citalopram [18]. The SSRIs as a class are now widely considered to be appropriate first-line anxiolytic drugs; in particular, paroxetine, one of the most potent 5-HT reuptake blockers, has been licensed in the United Kingdom for the treatment of each of the major anxiety disorders. Efficacy has been clearly demonstrated for SSRIs in randomized controlled trials of up to six months, but in common with other antidepressants research evidence is lacking for longer term efficacy.

A major advantage of the SSRIs is their improved tolerability relative to the TCAs and benzodiazepines, which has been demonstrated in comparisons of drugs from these classes [19, 20]. Nevertheless they are not without side effects: On initiation nausea, anxiety, jitteriness, and insomnia are related to the starting dose; later sedation, asthenia, headache, sweating, and sexual dysfunction may occur. Hyponatremia is a problem most likely to be seen in the elderly. Some effects are particular to individual drugs within the class [21]. For example, paroxetine has some anticholinergic properties and can cause dry mouth, constipation, and urinary hesitancy; sertraline is more likely to cause dyspepsia and diarrhea; and fluoxetine has agonist activity at 5-HT_{2C} receptors, causing headache, agitation, and loss of appetite, possibly explaining its efficacy in bulimia [22].

Although SSRI overdose can cause seizures, coma, and cardiac abnormalities [23], these toxic effects occur only in large overdoses or in combination with other drugs. Fatality rates are very substantially lower than with TCA overdose [24, 25]. Public attention has been drawn to reports of suicidal and aggressive thoughts and behavior associated with SSRI therapy [26]. The scientific basis for this assertion is disputed, and a number of conflicting factors have been considered [27, 28]. Both anxiety disorders and related depression significantly increase the risk of suicide and successful treatment

reduces this risk. However, there are periods within treatment when clinical factors can lead to an increased risk; for example, as energy levels improve, the patient may be more likely to act upon existing suicidal thoughts, and this phenomenon was observed before the advent of antidepressants. Furthermore there may be factors specific to antidepressant therapy that cause increased suicidality, such as side effects of agitation, restlessness, and increased anxiety which are possibly due to activation of postsynaptic 5-HT₂ receptors by increased synaptic 5-HT. These factors have been particularly highlighted as relating to the use of SSRIs but are in reality common to most antidepressants. The assessment and monitoring of suicidality are an integral part of the clinical management of any patient with anxiety or depression. It is debatable whether SSRI therapy presents a special risk in this area, and taking a broader view it appears that SSRI treatment is associated with reduced suicidality on a population level [29, 30].

A further controversy that has surfaced in the lay media is the misleading claim that SSRIs have “addictive” properties. This centers around reports of patients suffering symptoms when trying to discontinue medication. In many cases these symptoms are a recurrence of the original anxiety disorder, often as a result of suboptimal treatment, although an important difference between antidepressants and benzodiazepines is that rebound anxiety (a rapid return of the original symptoms) has not been clearly demonstrated with antidepressants. There is also a well-described transient discontinuation syndrome associated with SSRI withdrawal, that is, a reaction occurring as brain levels of the drug are falling that is not a feature of the underlying disorder [28, 31]. The most frequently occurring symptoms are dizziness, nausea, and headache (Table 2.3), with a typical duration of two to four days. The syndrome is most common with paroxetine, possibly due to its anticholinergic activity, and is very uncommon with fluoxetine due to the long half-life of its metabolites [32]. Although prominently highlighted with SSRIs, a similar syndrome has been observed with most, although not all, antidepressants. It can start as soon as 48 h after the final dose, and although most cases resolve within two to three weeks, symptoms may rarely last longer than this. Difficult cases can be managed by reinstituting the drug and withdrawing slowly, if necessary using liquid preparations, or by switching to a drug with a long half-life such as fluoxetine. Patients should be counseled about possible withdrawal problems at the start of therapy, although with careful management significant problems should not be common.

TABLE 2.3 SSRI Discontinuation Syndrome

Symptoms	Neurological symptoms: dizziness, tremor, vertigo, paraesthesia/shooting pains Psychological symptoms: anxiety, confusion, memory problems Somatic distress: nausea, headache, lethargy Hyperarousal: agitation, restlessness, insomnia, irritability
Risk factors	Treatment factors: longer duration of treatment, rapid discontinuation, short half-life drug, possibly increased dose Patient factors: possibly younger age, any psychiatric diagnosis
Therapeutic strategies	Careful assessment, reassurance, reinstitute therapy if necessary, taper slowly (over 1 month), switch to fluoxetine

SSRIs may interact with other drugs that have effects on 5-HT neurotransmission, including TCAs, buspirone, sumatriptan, and tryptophan, but particularly important is the interaction with MAOIs that can lead to a synergistic increase in synaptic 5-HT. This can result in the potentially lethal “serotonin syndrome,” comprising restlessness, irritability, tremor, sweating, and hyperreflexia [33]. In clinical practice there should be a washout of two weeks between discontinuing MAOI therapy and starting SSRI; a washout of one to two weeks should follow SSRI discontinuation (five weeks for fluoxetine). The drugs have variable potential for drug interactions via hepatic cytochrome P450 enzymes (Table 2.4). Citalopram and escitalopram have the lowest potential for interactions.

Expert sources and government guidelines recommend SSRIs as first-line drug treatments of anxiety disorders [34–37]. In preparation for treatment a full discussion of potential benefits and anticipated side effects (including discontinuation effects) should be held with the patient [38]. Some patients have difficulty initiating treatment because of anxiety about side effects. In these cases the drug may be increased slowly from a low starting dose, if necessary using the syrup form of fluoxetine or paroxetine, or a benzodiazepine may be used to cover the initiation period.

There is little research evidence to guide a decision on duration of treatment. Studies have shown continued improvement for up to 12 months, and for most disorders there is a significant relapse rate when treatment is stopped [39–41]. Guidelines suggest continuing for 12–24 months if treatment is successful, but a longer duration may be required for some patients (Table 2.5). Treatment disconti-

TABLE 2.4 SSRIs and Hepatic Cytochrome P450 Enzymes

Enzymes	Inhibitor of Enzymes
Citalopram/escitalopram	—
Fluoxetine	2D6 (potent) 3A4 (potent)
Fluvoxamine	1A2 (potent) 3A4 (potent) 2D6 (moderate)
Paroxetine	2D6 (potent) 3A4 (moderate)
Sertraline	2D6 (moderate)

TABLE 2.5 Patients Requiring Long-Term Treatment

Patient factors	Illness factors
Comorbid mood disorder	Severely disabling symptoms
Comorbid anxiety disorder	Chronic illness (> 2 years)
Physical ill-health	Recurrent episodes
Personality disorder	Incomplete response to treatment
Age > 50 years	

uation should be carefully planned and it is sensible to slowly taper off SSRIs as a matter of routine.

2.4.1.1.2 SNRIs. Venlafaxine is a serotonin and noradrenaline reuptake inhibitor (SNRI). It shares these properties with the TCAs amitriptyline, clomipramine, and imipramine, but it is the first selective SNRI with low affinity for muscarinic, histaminic, and α -adrenergic receptors. At low doses serotonergic effects predominate, but at higher doses (above 150 mg daily) the reuptake of noradrenaline is also significantly blocked [42]. It is not yet clear whether these noradrenergic effects confer additional benefit in the treatment of anxiety disorders. Venlafaxine is available as immediate- and extended-release (XR) preparations.

There is a large evidence base for the antidepressant efficacy of venlafaxine and increasing evidence for its use in anxiety disorders, including GAD [43], OCD [44], and social anxiety disorder [45], and for anxiety symptoms associated with depression [46]. Side effects on initiation of therapy are similar to those of SSRIs with nausea being the most common, and higher doses can cause elevation of blood pressure. A discontinuation syndrome similar to that seen with SSRIs has been reported. Toxicity causes cardiac conduction problems, seizures, and coma. There is a disputed suggestion that venlafaxine overdose may be associated with a higher mortality than that of the SSRIs [47]. This has led to the U.K. government recommending restriction of its prescription to secondary care [48], which appears a perverse decision when the mortality associated with venlafaxine overdose is substantially less than with other antidepressants, such as the tricyclics, which can be prescribed without restriction [25]. Although metabolized by the hepatic cytochrome CYP2D6, venlafaxine does not inhibit this enzyme and has a low potential for drug interactions.

Other SNRIs have been in development for some time and have recently been marketed on the basis of their antidepressant efficacy. As yet their efficacy in anxiety disorders is unproven, although the SNRI duloxetine was shown to be effective for anxiety symptoms associated with depression [49].

2.4.1.1.3 TCAs. This group includes compounds with actions on a range of neurotransmitter systems. Their antidepressant efficacy is primarily mediated by inhibition of the reuptake of 5-HT and noradrenaline, although side effects such as sedation may also have clinical benefits. Their efficacy in anxiety disorders is supported by a long history of clinical experience and a solid evidence base from controlled trials. Studies support the use of clomipramine (a potent 5-HT reuptake inhibitor) in panic disorder and OCD [50, 51], of imipramine in panic disorder and GAD [52, 53], and of amitriptyline in PTSD [54]. No controlled studies support the use of TCAs in social anxiety disorder.

A meta-analysis of controlled studies suggested superior efficacy of clomipramine over SSRIs in OCD [55], but this has not been demonstrated in direct comparisons, and the use of SSRIs has superseded that of TCAs because of advantages in safety and tolerability [56]. Side effects of TCAs include anticholinergic effects (drowsiness, dry mouth, blurred vision, and constipation), antihistaminergic effects (drowsiness and weight gain), and postural hypotension caused by α_1 -adrenoceptor blockade, as well as the side effects common to SSRIs. Some effects are dose related, and as the usual practice is to titrate the dose slowly upward, this often leads to patients being maintained on subtherapeutic doses. A discontinuation syndrome similar to that with SSRIs is well described, and withdrawal should be tapered. Overdose is associated with a significant

mortality due to hypotension, cardiac arrhythmias, metabolic acidosis, seizures, and coma [25]. Interactions can occur with other drugs with central nervous system (CNS) effects (particularly MAOIs), and with drugs that affect hepatic metabolism.

2.4.1.1.4 Mirtazapine. Mirtazapine has a novel mechanism of action that in theory should promote anxiolytic effects, although solid evidence from studies in anxiety disorders is awaited. It increases synaptic release of 5-HT and noradrenaline via blockade of presynaptic inhibitory α_2 -adrenoceptors as well as blocking postsynaptic 5-HT₂ and 5-HT₃ receptors and H₁ histamine receptors. Mirtazapine has good efficacy for anxiety symptoms associated with depression [57] and in controlled studies was superior to placebo in PTSD [58], equivalent to fluoxetine in panic disorder [59], and reduced social anxiety symptoms in heavy drinkers [60].

The actions of mirtazapine lead to a unique side-effect profile. Important effects are sedation, drowsiness, dry mouth, increased appetite, and weight gain. It does not cause initial worsening of anxiety. Tolerance to the sedative properties occurs after a few weeks, and paradoxically higher doses tend to be less sedating. Overdose does not cause serious toxic effects other than sedation. It has the potential for interaction with drugs that inhibit the cytochrome P450 2D6 and 3A4 isoenzymes, although reports of interactions are rare. Discontinuation symptoms have not yet been reported.

2.4.1.1.5 MAOIs. These drugs increase synaptic availability of 5-HT, noradrenaline, and dopamine by inhibiting their intracellular metabolism. The classical MAOIs phenelzine and tranylcypromine bind irreversibly to monoamine oxidase, while the newer drug moclobemide is a reversible inhibitor of monoamine oxidase A (RIMA). The long history of clinical use of MAOIs in panic disorder, PTSD, and social anxiety disorder is supported by several controlled trials [61–63], whereas the evidence for moclobemide is less conclusive. Both positive and negative studies have been published in panic disorder and social anxiety disorder, and meta-analysis suggests a lower response rate in social anxiety disorder than with SSRIs [64]. Brofaromine, a reversible MAOI, was effective in a controlled trial in social anxiety disorder but is no longer marketed.

The classical MAOIs have a significant side-effect profile, including dizziness, drowsiness, insomnia, headache, postural hypotension, and anticholinergic effects. Asthenia, weight gain, and sexual dysfunction can occur during long-term use. A hypertensive reaction (cheese reaction) may follow the ingestion of foods containing tyramine, which must therefore be removed from the diet. Overdose can be fatal due to seizures, cardiac arrhythmias, and hypotension. Interactions can occur with sympathomimetics, antihypertensives, and most psychoactive drugs, and a washout of two weeks is advised when switching from a MAOI to another antidepressant. Moclobemide is better tolerated, although at high doses (> 900 mg daily) its binding becomes less reversible and dietary restrictions should be observed. The main side effects are dizziness and insomnia. Overdose toxicity is less, although fatalities have been reported.

2.4.1.2 5-HT_{1A} Receptor Agonists. Buspirone is a partial agonist at postsynaptic 5-HT_{1A} receptors in the limbic system but a full agonist at autoreceptors in the raphe [65]. Acute dosage inhibits 5-HT release, but this recovers with continued administration.

It has anxiolytic effects that take several weeks to emerge, although in contrast to antidepressants it does not cause worsening of symptoms at initiation (Fig. 2.4). Buspirone is effective in the treatment of GAD [66] and for anxiety symptoms in depression [67], either as monotherapy or combined with an SSRI. Response is less favorable if the patient has recently taken a benzodiazepine [68]. Evidence is lacking to support the use of buspirone in other anxiety disorders. Other drugs in this class have demonstrated anxiolytic properties in preclinical studies. Gepirone is effective for anxiety symptoms associated with depression [69] and tandospirone is licensed for use as an anxiolytic in Japan.

Buspirone is well tolerated, particularly by the elderly [70]. Common side effects include dizziness, anxiety, nausea, and headache. It does not cause sexual dysfunction and does not appear to be associated with a discontinuation syndrome. Overdose causes drowsiness but not serious toxic effects. A potential for interaction with drugs that inhibit the cytochrome P450 3A4 isoenzyme is not a significant problem in clinical practice. GAD is usually a chronic condition and buspirone is suitable for long-term treatment. Patients should be advised to expect a slow onset of benefits and reviewed regularly in the early stages of treatment.

2.4.1.3 β Blockers. The rationale for using β -adrenoceptor blockers for the treatment of anxiety is twofold: first, for the control of symptoms caused by autonomic arousal (e.g., palpitations, tremor) and, second because of the postulated but poorly understood involvement of central noradrenergic activity in anxiety pathways. There is a history of clinical use of these drugs in each of the five major anxiety disorders, but evidence is lacking from controlled clinical trials, and positive findings have often been superseded by later negative studies. Early trials were carried out with propranolol and the more cardioselective atenolol, which has mainly peripheral effects. The efficacy of atenolol in performance anxiety suggests that not all of the effects are centrally mediated [71]. Recently there has been interest in pindolol, a β Blocker that also blocks 5-HT_{1A} autoreceptors and may promote serotonergic neurotransmission. Studies using pindolol to augment SSRI treatment of anxiety disorders have had mixed results [72–74].

Commonly β blockers cause side effects including bradycardia, hypotension, fatigue, and bronchospasm. Overdose can cause fatal cardiogenic shock. Because of the doubtful evidence for efficacy and poor tolerability and safety, their use in anxiety disorders is limited. They may have a circumscribed role in the prevention of performance anxiety [75].

2.4.1.4 Antipsychotics. This category contains drugs with various mechanisms of action that have clinical antipsychotic effects that are thought to be mediated via antagonism of D₂ dopamine receptors in the limbic system and cortex. They are loosely divided into two groups: older “classical” drugs such as haloperidol and chlorpromazine that are potent D₂ blockers and “atypical” antipsychotics that have a lower affinity for D₂ receptors but also block 5-HT₂ receptors. The history of clinical use of classical antipsychotics as “major tranquilizers” for anxiety disorders has little support from controlled trials [76]. Evidence is greatest in OCD for the augmentation of SSRI treatment with haloperidol [77] and the atypical drugs risperidone [78] and quetiapine [79]. Recent controlled trials have reported benefits for the atypical drug olanzapine in social anxiety disorder [80] and for olanzapine and

risperidone in addition to SSRIs in PTSD [81, 82]. Open studies are reporting efficacy for atypical antipsychotics in other anxiety disorders, and it may be that their clinical use expands in the future. The atypicals have advantages in tolerability and safety over the older drugs, particularly a lower incidence of extrapyramidal movement disorders, although they may cause sedation, weight gain, and metabolic effects. Their metabolism by cytochrome P450 enzymes leads to a potential for interaction with many coprescribed drugs.

2.4.2 Drugs Acting via Amino Acid Neurotransmission

Glutamate is the major excitatory amino acid in the brain and has a key role as a neurotransmitter in memory, learning, and stress responses. Glutamate receptors are present throughout the CNS but differ widely according to their localization and function [83], and as a result have not been easy to identify as targets for pharmacological manipulation (see also Chapters 10 and 11 in Volume I of this handbook). The role of glutamate in facilitating new learning has been established for some time, and it has recently been suggested that augmenting glutamate transmission may enhance recovery from anxiety disorders by accelerating the learning of new, nonanxious responses [84].

GABA is formed by the decarboxylation of glutamate and is the major inhibitory neurotransmitter (see also Chapters 12 and 13 in Volume I of this handbook). Recent research has centered on the role of the GABA_A receptor as the mediator of the anxiolytic and sedative effects of drugs such as alcohol and the benzodiazepines [85]. This complex transmembrane receptor consists of five linked subunits in a ring structure around a central chloride ion channel (Fig. 2.5). Binding of the endogenous neurotransmitter changes the configuration of the receptor, causing an increased permeability to chloride ions and inhibiting neuronal firing. Exogenous drugs bind to different sites on the receptor and affect its function, either in a direct fashion or by modulating the effects of GABA. The structure of the receptor depends on the configuration of its protein subunits, and nearly 20 subtypes have been found in the brain, with differences in topographical distribution and between individuals. Different receptor subtypes have different sensitivity to drugs such as alcohol and

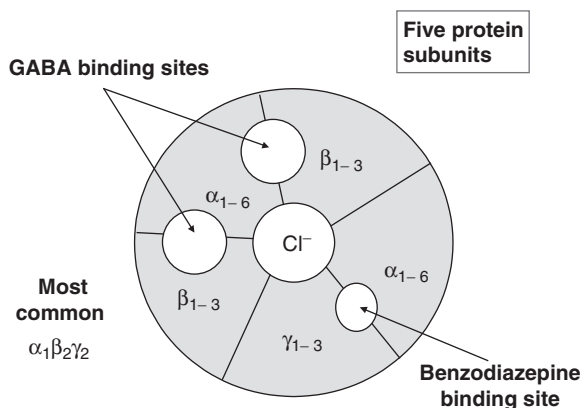


Figure 2.5 The GABA_A receptor.

may explain differences in the sensitivity of individuals to these drugs. Abnormalities of the GABA_A receptor have been identified in humans with anxiety disorders [86].

For most of the second half of the twentieth century the benzodiazepines were the mainstay of the treatment of anxiety. They have now been surpassed by antidepressants in terms of overall efficacy and tolerability, but despite concerns about their long-term safety, they remain an important therapeutic option. The group of anti-convulsants contains a number of drugs that act via GABA or glutamate neurotransmission and have a limited but interesting role in the treatment of particular anxiety disorders.

2.4.2.1 Benzodiazepines. The efficacy of benzodiazepines has been proven in anxiety disorders through extensive clinical experience and controlled trials [87, 88], although it is important to note that they are not effective at treating comorbid depression and there is less evidence to support their use in OCD and PTSD. Their anxiolytic effects have an immediate onset, and in contrast to other groups they do not cause a worsening of anxiety when therapy is initiated (Fig. 2.4). They are generally well tolerated, although side effects such as sedation, loss of balance, and impaired psychomotor performance may be problematic for some patients. There are reported associations with road traffic accidents [89] and with falls and fractures in the elderly [90]. They are relatively safe in overdose [91], although the risk is increased if taken in combination with alcohol or other sedative drugs.

The major controversy surrounding the use of benzodiazepines has concerned the risks of long-term treatment, specifically tolerance, abuse, dependence, and withdrawal effects. From being the most widely prescribed psychotropic drug they suffered a major backlash, although a more balanced view of their place in treatment has subsequently emerged [92] and they continue to be favored by patients and prescribers [93]. After 40 years of clinical experience there is little evidence of tolerance to the anxiolytic effects of benzodiazepines [94]. Abuse (taking in excess of the prescribed dose) is uncommon except in individuals with a history of abuse of other drugs, who may not be suitable for benzodiazepine therapy [95]. There is, however, a consensus that adverse effects on discontinuation are more common than with other anxiolytics [96]. A careful clinical assessment is indicated in this situation, as these effects may be caused by recurrence or rebound (recurrence with increased intensity) of the original anxiety symptoms.

A benzodiazepine withdrawal syndrome has been described in some patients discontinuing therapy (Table 2.6). Although potentially serious, it is generally mild and self-limiting (up to six weeks) but may accompany or provoke a recurrence of anxiety symptoms and cause great concern to the patient. As with any other treatment, the risks and benefits of benzodiazepine therapy should be carefully assessed and discussed with the patient. Monotherapy will not be first-line treatment for the majority of patients, but benzodiazepines offer a valuable option that should not be discounted.

The potential for interactions with other medications comes from two sources: the exacerbation of sedation and impaired psychomotor performance by other drugs also causing these effects and alterations in the hepatic metabolism of benzodiazepines by drugs that are either inducers or inhibitors of cytochrome P450 enzymes. The increased toxicity in combination with alcohol is mostly pharmacodynamic but may partly be due to the inhibition of metabolism of some benzodiazepines by high alcohol concentrations. Other drugs that may have additive effects on sedation include

TABLE 2.6 Benzodiazepine Withdrawal Syndrome

Symptoms	Hyperarousal: anxiety, irritability, insomnia, restlessness Neuropsychological effects: dysphoria, perceptual sensitisation, tinnitus, confusion, psychosis Autonomic lability: sweating, tachycardia, hypertension, tremor, dizziness Seizures
Risk factors	Treatment factors: treatment duration > 6 months/High dose/Short-acting drug/Abrupt cessation Patient factors: severe premorbid anxiety/Alcohol/substance use/disorder/Female/Dysfunctional personality/Panic disorder
Therapeutic strategies	Gradual tapering; switch to long-acting drug, e.g., diazepam; cover with secondary agent (anticonvulsant, antidepressant); cognitive behavioral therapy

TCAAs, antihistamines, opioid analgesics, and the α_2 -adrenoceptor agonists clonidine and lofexidine. Most benzodiazepines undergo oxidative metabolism in the liver that may be enhanced by enzyme inducers (e.g., carbamazepine, phenytoin) or slowed by inhibitors (sodium valproate, fluoxetine, fluvoxamine). Oxazepam, lorazepam, and temazepam are directly conjugated and are not subject to these interactions.

The specific clinical use of the numerous available benzodiazepines depends on their individual pharmacokinetic and pharmacodynamic properties. Drugs with a high affinity for the GABA_A receptor (alprazolam, clonazepam, lorazepam) have high anxiolytic efficacy, and these drugs may have a particular role in panic disorder where subsensitivity of GABA_A receptors has been demonstrated [97, 98]; drugs with a short duration of action (temazepam) are used as hypnotics to minimize daytime sedative effects; diazepam has a long half-life and duration of action and may be favored for long-term use or when there is a past history of withdrawal problems; oxazepam has a slow onset of action and may be less susceptible to abuse.

Guidance on the clinical indications for benzodiazepine therapy is available from various sources [35, 36, 95]. Long-term therapy is most likely to present problems with discontinuation and is usually reserved for cases that have proven resistant to treatment with antidepressants alone. Patients may benefit from a two- to four-week course of a benzodiazepine while antidepressant therapy is initiated, as this counteracts the increased anxiety caused by some drugs [99]. A benzodiazepine may be useful as a hypnotic in some cases of anxiety disorder and can be used by phobic patients on an occasional basis before exposure to a feared situation.

Benzodiazepines are additionally discussed in Chapter 3.

2.4.2.2 Anticonvulsants. There is an overlap between the clinical syndromes of anxiety and epilepsy. Panic disorder and PTSD can present with symptoms similar to temporal lobe seizures, alcohol and drug withdrawal states can cause both anxiety and seizures, and some drugs (e.g., barbiturates and benzodiazepines) act as both anticonvulsants and anxiolytics. Most anticonvulsants act via the neurotransmission of GABA or glutamate and in recent years have offered a promising field for the development of novel anxiolytic therapies [83, 100]. Although preclinical studies have demonstrated their anxiolytic properties, the evidence base in humans is less impressive

and in practice they are reserved for second-line or adjunctive therapy. Drug interactions mediated via hepatic enzymes are a significant feature of this group.

Gabapentin increases GABA activity by a mechanism that is unclear. It causes dose-related sedation and dizziness. It has been shown in controlled trials to be effective in social anxiety disorder [101] and to benefit some patients with panic disorder [102]. Pregabalin is a related compound that has recently demonstrated efficacy in GAD in a phase III study [103].

Lamotrigine blocks voltage-gated sodium channels and inhibits release of glutamate. A controlled study found efficacy in PTSD [104]. Important side effects include fever and skin reactions.

The historical use of sodium valproate for anxiety is poorly supported by clinical trials. A randomized study showed efficacy in panic disorder [105] and benefit has been reported in open studies in OCD and PTSD. The major side effects are tremor, nausea, ataxia, and weight gain and there is the potential for drug interactions via inhibition of hepatic enzymes.

No satisfactory randomized controlled trials have been published demonstrating the efficacy of carbamazepine in anxiety disorders, although it has a history of use as an anxiolytic in panic disorder and PTSD. It has an unfavorable side-effect profile (nausea, dizziness, ataxia) and multiple drug interactions due to induction of liver enzymes. Levetiracetam has anxiolytic activity in preclinical studies and was helpful for patients with social anxiety in an open study [106]. Tiagabine blocks neuronal uptake of GABA and has reported benefits in panic disorder and PTSD [107]. Topiramate has complex actions on GABA and glutamate and was found to be helpful for some symptoms of PTSD [108]. Vigabatrin inhibits GABA metabolism and has been shown to block provoked panic attacks in healthy volunteers [109].

2.4.3 Drugs with Other Mechanisms of Action

2.4.3.1 Antihistamines. The longstanding use in some countries of hydroxyzine, a centrally acting H₁ histamine receptor antagonist, is supported by positive findings in controlled trials in GAD [110–112]. Hydroxyzine promotes sleep and its anxiolytic effects have an early onset. Although it causes sedation, tolerance to this effect often occurs and effects on psychomotor performance are smaller than with benzodiazepines [113]. It is well tolerated and withdrawal effects have not been reported. Although the evidence for its efficacy is not large, hydroxyzine provides an option for some patients with GAD for whom standard treatments are unsuitable.

2.4.3.2 Lithium. Lithium is effective in the treatment of mood disorders. Its mechanism of action is unclear, but is likely to be via modification of intracellular second-messenger systems. There are no controlled trials demonstrating the efficacy of lithium in anxiety disorders, but there have been case reports of its use as an augmenting agent in panic disorder and OCD. Its high toxicity and poor tolerability limit its use in anxiety in the absence of a stronger evidence base.

2.5 PHARMACOTHERAPY FOR ANXIETY DISORDERS

An overview of appropriate drug treatments for each of the major anxiety disorders as well as for depression with prominent anxiety symptoms is shown in Table 2.7.

TABLE 2.7 Pharmacotherapy for Anxiety Disorders

Disorder	First-Line Treatments	Second-Line Treatments	Other Treatments	Augmenting Agents
Generalised anxiety disorder	Venlafaxine 75–150 mg, SSRI (e.g., paroxetine 20 mg, escitalopram, 10 mg)	Imipramine 150 mg, buspirone 15–60 mg	Diazepam 5–30 mg, hydroxyzine 200–400 mg	Benzodiazepines (e.g., diazepam 5–30 mg)
Obsessive-compulsive disorder	SSRI (e.g., fluoxetine 20–60 mg, fluvoxamine 50–300 mg, paroxetine 20–60 mg, sertraline 50–200 mg)	Clomipramine 150–250 mg	—	Haloperidol 5–15 mg, quetiapine 25–600 mg, risperidone 1–3 mg
Panic disorder	SSRI ± benzodiazepine (e.g., citalopram 20–60 mg, escitalopram 5–20 mg, paroxetine 10 mg daily increasing to max 50 mg daily)	Clomipramine 150 mg, imipramine 150 mg, phenelzine 30–60 mg, alprazolam 3–6 mg	Clonazepam 0.5–3 mg, diazepam 5–30 mg, lorazepam 2–6 mg, Gabapentin, 500–1000 mg	Benzodiazepines (e.g., diazepam 5–30 mg), pindolol
Post traumatic stress disorder	SSRI (e.g., paroxetine 20–50 mg, sertraline 50–200 mg)	Amitriptyline 150–200 mg, mirtazapine 30–45 mg, phenelzine 30–60 mg	Carbamazepine 250–500 mg, lamotrigine 100–200 mg, venlafaxine 75–150 mg	Olanzapine 2.5–5 mg
Social anxiety disorder	SSRI (e.g., paroxetine 20–50 mg), venlafaxine 75–150 mg	Phenelzine 30–60 mg	Clonazepam 0.5–3 mg, gabapentin 500–1000 mg, moclobemide 600 mg, olanzapine 2.5–5 mg	Benzodiazepines (e.g., diazepam 5–30 mg), buspirone 20–40 mg
Specific phobia	—	SSRI (e.g., paroxetine 20 mg)	Benzodiazepines (e.g., diazepam 5–10 mg)	—
Depression with concomitant anxiety	SSRI ± benzodiazepine (e.g., paroxetine 20–40 mg), mirtazapine 30–45 mg, venlafaxine 75–150 mg	Amitriptyline 75–150 mg, clomipramine 75–150 mg	—	Benzodiazepines (e.g., diazepam 5–30 mg)

Note: Doses are the suggested total daily dosage.

This overview represents the current view of the authors based on their interpretation of the published evidence and their extensive clinical experience.

2.5.1 Generalized Anxiety Disorder

GAD is a prevalent, chronic, disabling disorder that is comorbid with other anxiety or mood disorders in the majority of cases [114]. While it is a relatively new diagnostic concept, longitudinal studies have reinforced its validity [115]. The core symptoms are chronic worry and tension, and GAD frequently presents with somatic complaints such as headache, myalgia, or insomnia [116]. The diagnosis requires symptoms to be present for at least 6 months, although the duration of illness at presentation is usually much longer than this. The presence of comorbidity leads to a worse prognosis [117]. Cognitive behavioral therapy (CBT) is a structured psychological therapy usually delivered by a trained therapist on an individual basis over 10–15 weekly sessions. It aims to elicit erroneous thinking patterns that lead to anxious thoughts and to train the patient to challenge them and adopt new thinking strategies. CBT has been shown to be effective in GAD and should be considered if available [118].

Recommended drugs for GAD are antidepressants, benzodiazepines, buspirone, and hydroxyzine [114]. The use of antipsychotics is not supported by controlled trials and is discouraged due to their poor long-term tolerability. Pregabalin (related to the anticonvulsant gabapentin) was effective in preliminary trials and may be a future treatment option [103].

Recent evidence has brought about a shift in prescribing in GAD, and now the usual choice for first-line treatment will be an antidepressant. Suitable drugs include venlafaxine [43] and the SSRI paroxetine [119]. A nonsedating TCA such as imipramine could also be used if tolerated and where the risk of suicide is deemed to be low [53]. Little research is available to guide a decision on treatment duration, and a standard recommendation for anxiety disorders would be to continue therapy for at least 12 months following clinical improvement [34]. Buspirone is also appropriate for long-term therapy in the absence of comorbid depression [120].

Benzodiazepines are effective as monotherapy [53] but are rarely used as first-line therapy in this context because of their side-effect profile. They have a useful short-term role for the rapid control of anxiety symptoms or for the control of somatic symptoms such as muscle tension and insomnia, particularly in the early stages of antidepressant therapy. Hydroxyzine has a limited role but can be considered if other treatments are unsuitable [111].

2.5.2 Obsessive-Compulsive Disorder

OCD is a disabling disorder that runs a chronic or recurrent course ([121]; see also Chapter 6). It is diagnosed by the presence of obsessions (recurrent, intrusive thoughts, images, or impulses that are experienced as irrational and unpleasant) or compulsions (repetitive behaviors that are performed to reduce a feeling of unease). The symptoms are present for at least 1 h every day and cause impairment of important functions. Prevalence has been measured in various populations and is around 1–2%. Symptoms start as early as the first decade and have often been present in excess of 10 years at presentation [122]. Depression occurs in more than

50% of cases and there is significant comorbidity with other anxiety disorders, eating disorders, and tic disorders. Although classified with the anxiety disorders, OCD is distinct from the rest of this group in its epidemiological profile and neurobiology. In clinical terms OCD symptoms respond to drugs that enhance serotonergic neurotransmission but not to noradrenergic drugs and poorly to benzodiazepines.

The recommended first-line drugs for OCD are SSRIs and the TCA clomipramine [123]. The required dosage is generally higher than that required for other disorders (e.g., clomipramine 150–250 mg, paroxetine 40–60 mg) and SSRIs have advantages in safety and tolerability. Long-term treatment may be required. There is good evidence for the efficacy of CBT and there may be added benefits from combining psychological and pharmacological therapies [124]. In cases poorly responsive to SSRI treatment augmentation with the antipsychotics haloperidol, risperidone, or quetiapine has support from clinical trials, and addition of buspirone, lithium, and the serotonin precursor L-tryptophan has also been tried. In severe treatment-resistant cases the neurosurgical procedure stereotactic cingulotomy should be considered [125].

2.5.3 Panic Disorder and Agoraphobia

This is also a common, chronic, and disabling disorder with its peak incidence in young adulthood [35]. A panic attack is defined as the sudden onset of anxiety symptoms rising to a peak within 10 min. DSM-IV requires 4 of 13 defined symptoms to be present. The symptoms are physical symptoms corresponding to those caused by autonomic arousal and psychological symptoms (fear of a catastrophic event) and depersonalization/derealization (an altered perception of oneself or the world around). Panic disorder occurs when there are recurrent panic attacks, some of which are uncued or unexpected, and there is fear of having further attacks. Agoraphobia is present in around half of cases [3] and is a poor prognostic indicator. For some patients the anticipatory anxiety or agoraphobia may be considerably more disabling than the original panic attacks. Panic disorder is comorbid with episodes of depression at some stage in the majority of cases [126], with social anxiety disorder and to a lesser extent GAD and PTSD, and with alcohol dependence and personality disorder. Comorbidity results in increased severity and poor response to treatment. Panic disorder is associated with a significantly increased risk of suicide, and this is increased further by the presence of comorbid depression [127].

There is solid evidence for pharmacotherapy for panic disorder with SSRIs [128], the TCAs clomipramine and imipramine [50, 52] and the benzodiazepines alprazolam, clonazepam, lorazepam, and diazepam [129–132]. Therapy is likely to be required for a minimum of 12 months, and the favorable tolerability of SSRIs will usually lead to their choice as first line therapy. Patients with panic disorder are sensitive to drug effects, so a low initial dose may be used and titrated up or a benzodiazepine may be coadministered for the first 2–4 weeks. Stopping treatment is associated with discontinuation effects and an increased risk of relapse and should be approached with caution. CBT is an effective treatment for panic disorder, and additional benefits may be gained from combination therapy [133].

Other drugs effective in controlled studies include the antidepressants phenelzine [61], moclobemide [134], venlafaxine [135], mirtazapine [59] and reboxetine [14] and the anticonvulsants sodium valproate [105] and gabapentin [102].

2.5.4 Post traumatic Stress Disorder

This is another anxiety disorder that is common although underdiagnosed and is usually severely disabling [136]. The diagnosis is given when specific psychological and physical symptoms follow exposure to a traumatizing event that invokes fear, horror, and helplessness. Symptoms fall into three categories: reexperiencing phenomena (flashbacks, nightmares, distress when memories of trauma are triggered), persistent avoidance of triggers to memory of the trauma and general numbing, and hyperarousal (insomnia, irritability, poor concentration, hypervigilance, increased startle response). Symptoms must persist for more than one month after the trauma. PTSD is highly comorbid with depression [137] and substance use disorders and is associated with previous exposures to trauma and a previous history of anxiety disorders. PTSD carries the highest risk of suicide among the anxiety disorders [138]. Without effective treatment it generally runs a chronic, unremitting course.

The evidence base for pharmacotherapy is shallow although improving. Efficacy is established for the SSRIs paroxetine [139], fluoxetine [140], and sertraline [141] and the TCA amitriptyline [54]. Treatment is started at standard dose but may be titrated upward (e.g., paroxetine 20–50 mg). Results from long-term studies are awaited, but treatment should be continued for a minimum of 12 months. Medication is given alongside psychotherapy, usually cognitive and exposure therapies [142]. Other treatments include the antidepressants phenelzine and mirtazapine, the anticonvulsants lamotrigine, sodium valproate, carbamazepine, and tiagabine, and augmentation with the atypical antipsychotic olanzapine. The use of benzodiazepines is not advised as their efficacy is not established and withdrawal symptoms may be particularly distressing. If insomnia is problematic, then a nonbenzodiazepine hypnotic may be prescribed.

2.5.5 Social Anxiety Disorder (Social Phobia)

This disorder is characterized by anxiety symptoms in social or performance situations accompanied by a fear of embarrassment or humiliation. Situations are avoided or endured with distress. There may be a specific fear of one or two situations (most commonly public speaking) or of three or more situations in the generalized subtype. Epidemiological studies find this to be the most prevalent anxiety disorder among the general population [143]. Peak onset is around the time of adolescence and the resulting impairments can have a profound effect on social and occupational development. If untreated it tends to follow a chronic, unremitting course. Social anxiety disorder is frequently comorbid with depression, other anxiety disorders, alcohol problems, and eating disorders. It is associated with an increased rate of suicide that is significantly higher in the presence of comorbidity [144].

Drug treatment studies have focused on the generalized subtype [145]. The largest evidence base is for the SSRIs, which are accepted to be the drug treatment of choice. Treatment is started at standard dose and increased as necessary (e.g., paroxetine 20–50 mg). Duration of treatment is usually for at least 12 months, and there is benefit from combination with CBT [146]. The other class of antidepressant to be considered is the MAOIs, as phenelzine and moclobemide have controlled trial data to support their use [63]. TCAs have no proven efficacy and evidence for venlafaxine and mirtazapine is awaited. Among the benzodiazepines only clonazepam has been

shown to be effective as monotherapy, possibly due to its effects on 5-HT_{1A} receptors [147]. Benzodiazepines may also be used to augment SSRI treatment. Other drugs to consider are the anticonvulsant gabapentin and the antipsychotic olanzapine. The β blockers are not effective in generalized social anxiety disorder but have a role in symptomatic control in specific performance anxiety.

2.5.6 Specific Phobia

In this disorder the patient has an inappropriate or excessive fear of a particular stimulus or situation, such as animals, heights, or thunder. An anxiety reaction is consistently and rapidly evoked on exposure to the stimulus, and there is anticipatory anxiety. Population studies have found a surprisingly high prevalence and associated disability, for example a lifetime prevalence of 12% in the National Comorbidity Survey [143]. The standard treatment for specific phobia is behavioral therapy, and patients rarely require pharmacological treatment. Nevertheless there are clinical and pharmacological similarities between patients with specific phobias and those with other anxiety disorders, particularly panic disorder [148], and it might be predicted that anxiolytic medications would have beneficial effects. A small controlled study found an improvement in measures of fear and avoidance after a four-week trial of the SSRI paroxetine [149], and there is also a role for the use of a short-acting benzodiazepine to control anxiety prior to exposure to the feared stimulus. A further interesting finding with potentially wider application is that the benefits of psychological therapy for height phobia were increased by the use of D-serine, a drug that enhances *N*-methyl-D-aspartate (NMDA) glutamate receptor functioning [150]. This may have been due to the effects of this drug in promoting learning and “over-writing” of conditioned phobic responses.

2.5.7 Anxiety Symptoms in Depressive Disorders

The prevalence of depression in patients with anxiety disorders is high, as is the prevalence of anxiety in patients with depression [151, 152]. Among patients presenting for treatment of anxiety symptoms, a large proportion will have a primary diagnosis of depression. In these situations it is critical to offer a treatment plan that will prove effective against both anxiety and depression [153]. The presence of both disorders together causes an increase in disability, increased severity of symptoms, a higher likelihood of suicidal thoughts, and a poor response to treatment [154].

Antidepressants would be the obvious drug class to select in this patient group, and a number of controlled studies have demonstrated their efficacy. Both SSRIs and TCAs are effective, with the most evidence being for the SSRI paroxetine and the TCAs clomipramine and amitriptyline [155–157]. Comparative studies favor the SSRIs because of their better tolerability, and safety is also a factor in a group at high risk of suicide. Recent studies have demonstrated the efficacy of the new antidepressants venlafaxine [46] and mirtazapine [57] in this group, and as their tolerability matches that of the SSRIs, they should also be considered as first-line treatment. Benzodiazepines produce a rapid improvement in anxiety but are ineffective at treating depression [158] and are not suitable for long-term treatment in this context. They have a short-term role on initiation of antidepressant therapy in selected patients.

2.6 CONCLUSIONS AND FUTURE DIRECTIONS

We have demonstrated that the recent shift in clinical practice toward the use of antidepressants, particularly SSRIs, for the first-line treatment of anxiety disorders is supported by research evidence from randomized controlled trials. Although there has been some negative reporting of the use of SSRIs, the overall situation with regard to anxiety disorders remains one of underdiagnosis and undertreatment, so it should be expected that SSRI use will continue to increase. Clinical practice may be refined in future years as important gaps in the current knowledge base are filled, including the optimal duration of treatment, the identification of patients at particular risk of relapse, the benefits of combining drugs with psychotherapy, and suitable options for patients resistant to first-line treatments. Health policy in the United Kingdom appears to be shifting toward the management of anxiety disorders in primary care [37], and this may potentially lead to an increase in provision of psychological therapies and further raise the profile of anxiety disorders.

New drugs available for the treatment of depression may also prove to be effective for anxiety disorders. In the immediate future these will be drugs acting on the monoamine system. While the prime position of the SSRIs has been reinforced by evidence for the role of 5-HT in anxiety, newer antidepressants such as the SNRIs and mirtazapine have a dual action on serotonergic and noradrenergic neurotransmission. This dual action appears to confer additional benefits in the treatment of depression, but it is not yet clear whether the same is true in anxiety disorders. Further clinical studies are required to fully define the role of venlafaxine and mirtazapine, and clarification of the role of noradrenaline in anxiety is likely to occur.

Many other drug groups with actions on monoaminergic neurons have demonstrated anxiolytic properties in preclinical studies and may in the future prove to be effective treatments. The most likely candidates at present are the azapirones, which are partial agonists at 5-HT_{1A} receptors and have already demonstrated antidepressant and anxiolytic activity in humans [65]. Other potential candidates include the 5-HT_{2C} receptor antagonist and melatonin receptor agonist agomelatine [159], other 5-HT_{2C} antagonists [160], 5-HT_{1B} receptor agonists and antagonists [161] and 5-HT₄ antagonists [162].

It is only in recent years that drugs acting via GABA neurotransmission have been supplanted as first-line treatments, and new drugs in this class with improved tolerability compared to the benzodiazepines are likely to be marketed in the near future [163]. Other GABAergic drugs already marketed for their anticonvulsant properties, such as the selective GABA reuptake inhibitor (SGRI) tiagabine, may prove to have useful efficacy in anxiety disorders [164]. Further down the line agonists that are selective for specific subunits of the GABA_A receptor offer the prospect of drugs that are anxiolytic but with fewer sedative properties [165].

Overall it is remarkable that current pharmacological strategies are centered around such a small number of brain mechanisms. Future strategies may involve glutamate neurotransmission, particularly inhibition of mGlu5 receptors [166], and neuropeptides such as corticotropin-releasing factor antagonists ([167]; see also Chapter 5) and substance P antagonists [168, 169]. A continued expansion in the range of anxiolytic therapies should be anticipated.

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BENZODIAZEPINES

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3.1	Introduction	93
3.2	Pharmacology of Benzodiazepine Receptor Ligands	97
3.2.1	Therapeutic Action of BZ Receptor Ligands	97
3.2.2	Endogenous Benzodiazepine Site Ligands	98
3.2.3	Modulation of Single-Cell GABA Response by Benzodiazepines	99
3.2.4	Tolerance and Dependence to BZ	100
3.2.5	Metabolism of BZ Receptor Ligands	101
3.3	GABA _A /Benzodiazepine Receptors	103
3.3.1	Subunit and Subtype Structural Diversity	103
3.3.2	Functional Domains	105
3.3.2.1	GABA and BZ Binding Pocket	105
3.3.2.2	Assembly, Clustering, and Surface Expression	108
3.3.3	Diversity of Brain Distribution	110
3.3.4	BZ Functional Diversity as Revealed by Gene Knockout and Knockin Models	112
3.4	Structure Activity Relation of Benzodiazepines	114
3.5	Future Developments	116
	Acknowledgment	117
	References	117

3.1 INTRODUCTION

The widespread use of benzodiazepines (BZs) is largely due to their powerful anxiolytic and hypnotic properties in combination with safety even at high dosages when used alone without other central nervous system (CNS) depressant drugs or alcohol. Strictly speaking, only the 1,4- and 1,5-benzodiazepines (Figs. 3.1–3.3) belong to this class of compounds. But since the development of chlordiazepoxide (see below), a large number of chemically unrelated groups of tranquilizers have been

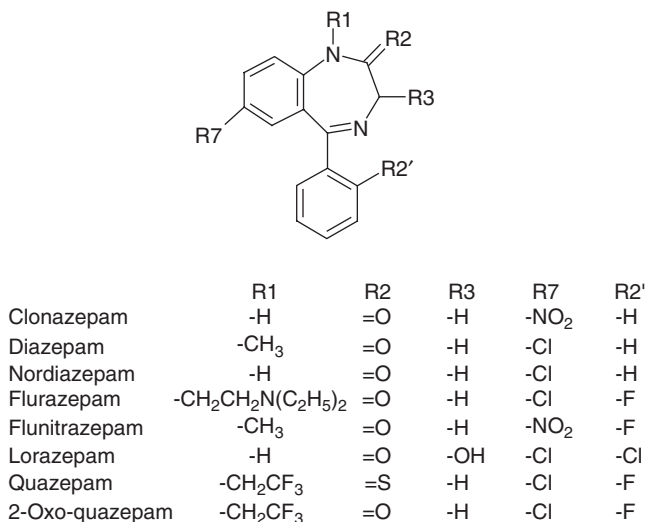


Figure 3.1 Structure of 1,4-benzodiazepines. Shown are several subtype non-selective 1,4-benzodiazepines as well as two ligands with some preference for α_1 -containing GABA_A/BZ receptors (quazepam and 2-oxoquazepam).

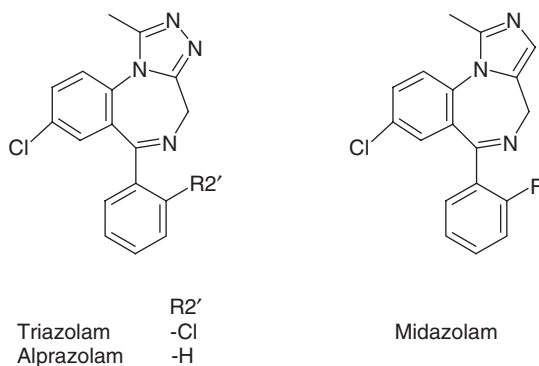
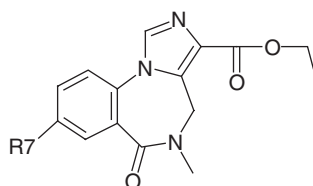


Figure 3.2 Structure of two triazolo-1,4-benzodiazepines and midazolam. All compounds shown are non-selective to various GABA_A/BZ-receptor subtypes.

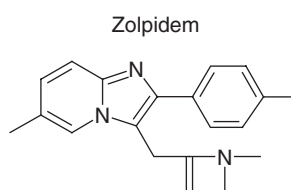
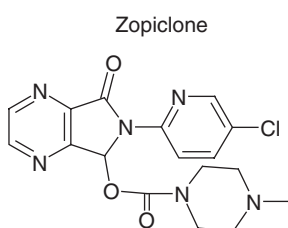
synthesized which act with high affinity and specificity via the BZ receptor sites. Among the most important classes are the triazolopyridazines (e.g., CI 218,872; Fig. 3.4), imidazopyridines (e.g., zolpidem; Fig. 3.4), and β -carbolines (e.g., β -carboline-3-methylester; Fig. 3.4).

The first known and intensively studied synthetic tranquilizers were the barbiturates meprobamate, reserpine, and chlorpromazine. In an attempt to circumvent the molecular manipulation approach and to obtain a novel chemical type of tranquilizer, Leo H. Sternbach and colleagues, who developed the first BZ compounds, did not start from a biochemical or pharmacological point but from the viewpoint of a chemist in that

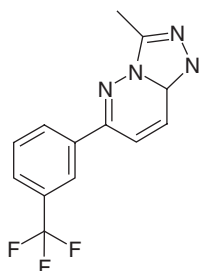


	R7
RO15-4513	-N ₃
Flumazenil	-F

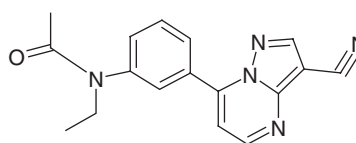
Figure 3.3 Structure of two imidazo-1,4-BZs. Whereas RO 15-4513 is a partial negative modulator on α_1 -, α_2 -, α_3 -, and α_5 -containing receptors and recognizes all GABA_A/BZ receptors with high affinity, flumazenil is an antagonist and binds with high affinity only to α_1 -, α_2 -, α_3 -, and α_5 -containing receptors and with a much lower affinity to α_4 - and α_6 -containing receptors.



CL 218,872



Zaleplon



β -Carboline-3-methylester

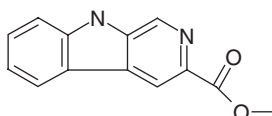


Figure 3.4 Structure of some non-BZ ligands of GABA_A/BZ receptor. CL 218,872 is the prototypical α_1 -preferring ligand. As well, β -carboline-3-methylester is a long-known α_1 -preferring ligand, but in contrast to CL 218,872, it is a full negative modulator. Zolpidem is the most selective GABA_A/BZ receptor ligand known binding with high affinity only to α_1 -containing receptors and with moderate affinity to α_2 - and α_3 -containing receptors. Zaleplon and zopiclone are less selective than the former two but still do not display the full functionality of the classical 1,4-BZs.

he postulated that the structures should be more or less unexplored, be easily obtainable, and be demanding for a synthetic chemist [1]. Even more so, their discovery was not the planned synthesis of a given structure but the result of an unexpected chemical reaction. Thus, the structure of the first in this series of tranquilizers, generically named chlórdiazepoxide and marketed as Librium, was only established some time after its synthesis and after it had been shown to be an efficient minor tranquilizer.

Twenty years after the first human in vivo pharmacological testing the molecular targets of BZ ligands were simultaneously described by three groups using [^3H]diazepam as a tool [2–4]. Since then, research into the action and function of BZ has taken advantage of the ease with which the ligands can be measured in vivo and in vitro using their tritiated forms. The novel selective labeled ligands, in conjunction with the development of molecular biology techniques, paved the ground to study the biochemical and neurobiological functions of the BZ in such detail that they are now among the best understood neuropharmacological agents.

Even before the BZ binding sites were described on a molecular level, it was speculated that γ -aminobutyric acid (GABA) plays a crucial role in the central action of BZ [5, 6]. Gradually, a picture evolved in which neuronal BZ binding sites were physically coupled to GABA type A (GABA_A) receptors to form the GABA_A /BZ receptor complex [7–9]. This model was further proven by demonstrating that affinity-purified BZ receptors contain sites for the specific GABA_A agonist [^3H]muscimol [10]. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) the purified protein separated into two major bands, labeled α (50 kDa) and β (57 kDa). By irreversibly photolabeling BZ receptors with [^3H]flunitrazepam the 50-kDa band is strongly labeled, but tissue-specific heterogeneity of the central BZ receptor was demonstrated by showing that other than the α and the β bands specifically incorporated [^3H]flunitrazepam [11]. Only the introduction of the molecular binding techniques finally settled the issue and started a new area in BZ ligand development and GABA_A /BZ receptor research.

Shortly after the discovery of BZ binding sites in the CNS, specific high-affinity [^3H]diazepam binding was described in various peripheral tissues and in transformed cells of neuronal origin [4, 12]. These sites are physically and pharmacologically distinct from sites present in tissues derived from the neuronal crest during ontogeny [13–15]. Though diazepam (Fig. 3.1) and most other clinically important BZs [16] bind with high affinity to both sites, other BZ ligands preferentially recognize one or the other: The 4'-chloro-substituted diazepam (RO 5-4864) and the isoquinoline Pk 11195 recognize only the “peripheral-type” site, while flumazenil (RO 15-1788, Fig. 3.3) and clonazepam (Fig. 3.1) are specific ligands for the “central-type” BZ site. The distribution of the “central-type” receptors made them likely candidates for the therapeutic action of the BZs; the peripheral sites were originally termed “acceptors” to denote a lack of physiological or pharmacological function [17]. Since then, the peripheral-type BZ receptor protein was shown to be located in the outer mitochondrial membrane [18] and to be most likely involved in cholesterol uptake and steroid exchange across that membrane [19, 20]. It is an 18-kDa protein with five transmembrane α -helical domains [see 21], clearly different from subunits of the GABA_A receptor. This BZ recognition site is unlikely to be involved in the central action generally associated with BZ. Therefore, the remainder of the text will concentrate on the GABA_A /BZ receptors. For a more detailed discussion on the structure of GABA receptors, see Chapter 12 in volume I of this handbook.

3.2 PHARMACOLOGY OF BENZODIAZEPINE RECEPTOR LIGANDS

3.2.1 Therapeutic Action of BZ Receptor Ligands

The 1,4-BZs exhibit remarkably similar clinical profiles, demonstrating anxiolytic, sedative, myorelaxant, anticonvulsant, amnestic, and respiratory depressant properties. However, there is a separation in the doses needed to achieve these effects, though the size of the therapeutic window differs slightly between different BZs. Only when it comes to receptor subtype selective compounds can sedation be more prevalent than any of the other effects (see Fig. 3.5).

A large number of new BZ receptor ligands have been evaluated preclinically and clinically in recent years, all of them structurally different from the classical 1,4- and 1,5-BZs. These compounds include the hypnotic agents zolpidem, zaleplon, and zopiclone (Fig. 3.4). The imidazopyridine zolpidem is a highly potent sedative and hypnotic [22]. In contrast to the classical BZ it does not seem to alter sleep architecture or induce rebound insomnia after discontinuation [23, 24]. Zolpidem decreases sleep latency and increases sleep duration and reduces the number of awakenings [25]. Zolpidem has rapid metabolism with metabolic elimination $T_{1/2\beta}$ values of 1.5–3 h, and when used at normal evening doses to treat insomnia, the residual cognitive impairment effects the next morning are negligible. While zaleplon may be best indicated for the delayed onset of sleep, zolpidem and zopiclone may be better indicated for maintaining a complete night's sleep [26]. For a detailed discussion on the hypnotic properties of benzodiazepines, see Chapter 6 in volume III of this handbook.

BZs are relatively safe, even in large doses. However, especially when administered together with other sedative substances, that is, ethanol, barbiturates, or opiates, toxic effects may result. These include general apathy, muscular atonia, ataxia, and inhibition of the respiratory system. Under severe intoxication flumazenil can be given as an antidote, though care must be taken as this compound has a much shorter

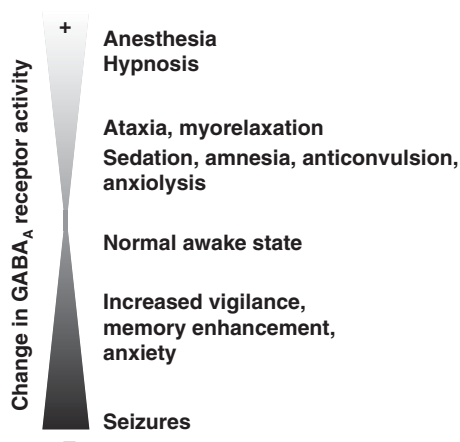


Figure 3.5 Correlation of activity at GABA_A receptors with clinical effects. The intrinsic activity of BZ receptor ligands ranges on a continuum from full positive (top) to full negative modulators (bottom). Receptor-subtype-specific compounds with the less than full efficacy may avoid unwanted GABA-mediated effects.

half-life than many other BZ ligands in clinical use, which might make the repeated administration necessary.

3.2.2 Endogenous Benzodiazepine Site Ligands

Ever since the discovery of the BZ receptors, scientists have been interested in finding endogenously produced substances that would act naturally via these binding sites and would be called endozepines. Many substances have been described that interfere with BZ binding [27–29], but a definitive proof of physiological significance of any of these compounds is still missing. Without going into details of each putative compound, a few of them are still being investigated and may finally prove to be active endogenous BZ ligands, at least in certain pathophysiological conditions.

It has been clearly demonstrated that 1,4-BZs are present in the body and brain, even in postmortem brains from patients deceased 20 years before the first BZs have been synthesized [30, 31], suggesting that, for example, *N*-desmethyldiazepam could be formed by endogenous enzymes. These pathways have been poorly characterized in humans, and, therefore, the compounds might originate from plants or bacteria known to be able to synthesize BZs. As well, BZ ligands with negatively modulating properties may be endogenously formed from the β -carboline series of compounds [32], although some of the early compounds isolated from urine may not be present in brain or be artifacts of the isolation procedure [33, 34].

One of the most interesting cases has been the discovery of a peptide of about 100 amino acids called diazepam binding inhibitor (DBI). DBI was suggested to act as an endogenous inverse agonist at GABA_A/BZ receptors [35, 36]. It is a competitive inhibitor of [³H]flumazenil binding to central GABA_A receptors and to [³H]RO 5-4864 binding to peripheral-type mitochondrial BZ receptors [37]. It is present in several different forms and mostly in brain glial cells and tumors and in peripheral tissues [38, 39]. Interestingly, DBI was found to be a member of the acyl-coenzyme A (CoA) binding protein family (ACBP [40, 41]), which have been implicated in many cellular functions including the modulation of acyl-CoA concentrations within cells and are considered products of widely conserved housekeeping genes. The gene also contains a sterol regulatory element [42], which allows DBI/ACBP to be regulated with other genes affecting lipid metabolism. DBI/ACBP has also been shown to activate steroidogenesis by facilitating cholesterol transport to the inner mitochondrial membrane, a process mediated by the peripheral-type BZ receptor [43, 44]. Thus, DBI is a ligand for the mitochondrial BZ receptor but might not be so relevant as an endogenous BZ acting on the central GABA_A receptors. Since various BZ molecules have very variable affinities to the peripheral sites (see above), the behavioral and physiological actions of all these drugs cannot be mediated by these sites.

Stupor associated with hepatic encephalopathy has been treated with flumazenil [45–47], and while amelioration of the symptoms has been observed, it is apparently not so effective to become the only routine therapy [48]. Its efficacy indicates increased amounts of endogenous BZs in these patients, which has been confirmed both in patient samples and from tissues of animals with hepatic failure. The identities of the increased endogenous ligands are not yet settled, but they include BZ like structures and DBI-like peptides [49–52]. Furthermore, in acute intermittent porphyria there are increased concentrations of hemoglobin metabolites, of which hemin and protoporphyrin IX have been shown to potentiate at micromolar

sensitivity the GABA responses of the $\alpha_1\beta_2\gamma_2$ GABA_A receptors via flumazenil-sensitive BZ sites [53]. Interestingly, the latter compounds are also known to have even higher affinities toward the guinea pig brain peripheral-type BZ receptors [54].

The physiological relevance of the naturally produced BZs or other endogenous ligands still remains unclear, but from the experimental point of view it should be kept in mind that the recent BZ site point-mutated mouse models (see below) have not revealed clear behavioral or physiological alterations without drug challenges. This fact does not support any major role for the endogenous BZ site ligands in normal brain function, although they might be involved in pathological conditions, such as hepatic encephalopathy.

3.2.3 Modulation of Single-Cell GABA Response by Benzodiazepines

Detailed analysis of single-channel kinetics confirmed early reports [55] that BZs do not affect GABA-induced single-channel conductance or the average channel open duration [56–58] but increase channel-opening frequency by elevating the number of bursts [56, 57]. Zolpidem has little or no effect on mIPSC frequency, rise time, or amplitude but causes a significant prolongation of the miniature inhibitory postsynaptic current (mIPSC) decay [59]. The negative modulatory β -carboline DMCM (compare β -CCM in Fig. 3.4) reduces the channel-opening frequency without altering open duration or channel conductance; that is, it behaves inversely to positive modulating BZ [58]. BZ potentiation of the GABA response is discussed as originating from an increased affinity for GABA (Fig. 3.6). In this case, BZ should increase the average channel open duration as is observed with increased GABA

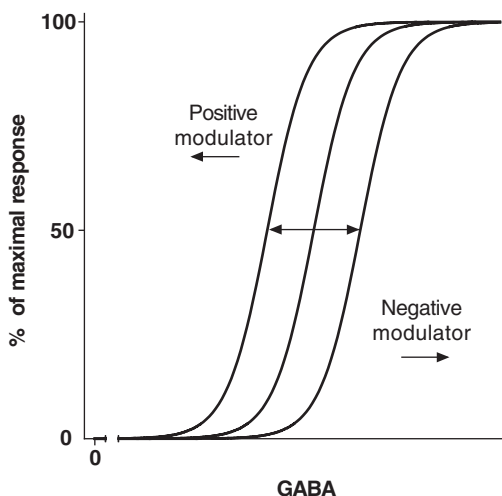


Figure 3.6 Effect of positive and negative modulators at BZ recognition site. Positive modulators shift the dose–response curve of GABA at a given GABA_A receptor subtype to the left, whereas negative modulators right shift the curve. At the level of spontaneous inhibitory postsynaptic currents, the modulators mainly affect the current decay. Neither class has any effect on the maximal response of GABA or any effect in the absence of GABA, that is, the endogenous neurotransmitter controls the postsynaptic effect, unless the synaptic GABA release fails to saturate the postsynaptic receptors.

concentrations [56], but kinetic analysis [57] does not support such a mechanism [58]. The increased channel-opening frequency might be explained by an increased affinity at only one of multiple binding sites, different transitions into desensitized states, or altered coupling between binding site and channel [56].

In view of the close to saturating concentrations of GABA in the synaptic clefts of most neurons, BZ receptor ligands might exert their effects mainly by an increase in the decay time constants, thus prolonging the action of GABA [60]. In some neurons, the number of GABA_A receptors in their synapses differ and BZ can increase the mIPSC amplitudes in those synapses with a high number of receptors apparently not saturated by released GABA, while no amplitude potentiation takes place in the synapses with low GABA_A receptor number [61]. BZs can also act on extrasynaptic receptors (e.g., [62]), where the surrounding GABA concentrations are insufficient to cause the maximal effect, although quite a number of extrasynaptic receptors are BZ nonsensitive as they lack the γ_2 subunits [63].

The actions of BZ on GABA_A receptors at single receptor and synapse levels must finally be converted to effects on activities of neuronal pathways and circuitries, which then affect widespread physiological and mental processes.

3.2.4 Tolerance and Dependence to BZ

The GABAergic system has been implicated in the mechanisms of drug abuse, in the abuse not only of BZ-positive modulators but also of other drugs of abuse, such as ethanol, opioids, cannabinoids, nicotine, and stimulants [64]. This is of no surprise, since the GABAergic system is so widespread and regulates most brain systems. Furthermore, novel mechanisms via GABAergic pathways have been proposed to be responsible for the rewarding actions of GABA_A receptor-positive modulators and antagonists after intracerebral injections into different regions of ventral tegmental and caudal hypothalamic regions [65–67]. Effects of chronic use leading to tolerance and abuse to BZ compounds can be examined at the levels of GABA_A receptor sensitivity, receptor subunit alterations, and counteracting adaptations in other neuronal systems.

Chronic treatment of experimental animals or neuronal cell cultures with BZ agonists have shown that the receptor binding determined by various ligands does not alter much [68, 69], suggesting that GABA_A receptor subunit levels are rather stable even during continued receptor stimulation. Similar disappointing results have been obtained when receptor subunit gene transcription has been evaluated by subunit-specific oligonucleotide probes for messenger RNA (mRNA) [70], indicating that the receptor subunit synthesis remains mostly stable. In addition, subunit switches, such as a general change in dominance from γ_2 to δ subunit-containing receptors, have been excluded [70]. Still, there has been a more consistent finding of reduced GABA sensitivity after BZ treatment, especially a reduced or even abolished GABA stimulation of [³H]BZ binding [68, 71], indicating that, while the binding sites are intact, they are in a state where allosteric interactions can no longer facilitate receptor function. Along this line, a similar loss of potentiation after chronic BZ treatment has been observed in the GABA actions stimulated by BZ [68]. It is possible that the reduced coupling between GABA_A receptor binding site domains by chronic treatments is due to altered posttranslational processing of receptor subunits/subtypes and/or by receptor endocytosis, as it can take place rapidly within a few hours [72].

On the other hand, some research groups have been able to find defined alterations in brain regional GABA_A receptor subunit expression, which might correlate with the emergence of tolerance to various BZ agonists. In frontoparietal motor and somatosensory cortex of rats, at least α_1 -subunit expression decreased and that of α_3 , α_5 , $\beta_{2/3}$, and γ_2 subunits increased in a region-selective manner during a 14-day diazepam treatment that resulted in tolerance [73]. The suggested antipanic compound alprazolam alters the expression of brain stem α_3 -, β_1 -, and γ_2 -subunit expression in rats [74]. A defined reduction in the hippocampal α_5 -subunit expression in mice has been suggested to be mainly responsible for the tolerance development to low doses of diazepam [75], in keeping with earlier experiments showing reduced binding of the α_5 -subunit-selective ligand [³H]RY-80 in the hippocampal CA1 region in rats after flurazepam treatment [76]. Thus, it seems that there are several receptor-subtype-dependent mechanisms for tolerance development, partly depending on the animal species and behavioral assays used for tolerance assessment, and conditioning mechanisms, as hinted by the involvement of the hippocampal receptor subtypes. These mechanisms might thus be brain region specific. However, we also need more receptor-subtype-selective functional studies to understand the brain pathways involved in the BZ tolerance mechanisms.

The apparent diversity of the mechanisms accounting for BZ tolerance is further complicated by adaptations in other mechanisms than the GABA_A receptor. Acute diazepam administration at low sedative doses induces the expression of several genes in the cerebral cortex of mice, including growth factors, such as brain-derived neurotrophic factor, and transcription factors and kinases [77]. Interestingly, calcium/calmodulin-dependent protein kinase II remained upregulated at least 40 h after a single diazepam dose. All these changes were dependent on the α_1 -subunit-containing receptors as they were absent in α_1 (H101R) knockin mice. One of the most interesting findings has been the increased function and protein levels of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors in the hippocampus during chronic treatment with flurazepam or diazepam [78–80]. Especially the GluR-A (GluR-1) subunit of AMPA-type glutamate receptors has been upregulated in certain brain regions by many other drugs of abuse, such as morphine, cocaine, and amphetamine [81–84]. This indicates that the AMPA receptor facilitation, upregulation, and/or increased cell surface targeting are not specific for BZ tolerance or withdrawal. These non-GABA_A receptor adaptations might become important targets for reducing tolerance, dependence, and withdrawal phenomena to BZs and other drugs of abuse.

Most of the dependence experiments have been carried out with long-acting BZ ligands, such as diazepam and flurazepam. However, all full agonists, independent of the duration of action, possess the property of inducing tolerance and dependence. Importantly, the partial agonists so far studied usually do not induce strong tolerance or withdrawal symptoms at experimentally relevant doses [85–88]. However, as none of these has passed into clinical use, we can only refer to these reviews on these compounds.

3.2.5 Metabolism of BZ Receptor Ligands

As detailed before, the pharmacological action of all chemically related BZs in clinical use is similar with the exception of the antagonist flumazenil, though minor variations in the affinity and efficacy between the various subtypes have been

reported. As these minor differences in the receptor actions between the ligands are unlikely to explain all observed clinical variances, they may be mainly or even exclusively due to differences in the absorption and metabolism of the drugs. As even the absorption is close to complete for most of the BZ receptor ligands of this chemical class, most differences can be traced back to the respective metabolic paths. After oral administration the time to peak levels detected in the plasma ranges from 30 min (~ 1 h for diazepam) up to 8 h. As well, the biological half-life of BZ varies greatly between a few hours (e.g., alprazolam, brotizolam) and more than 20 h (e.g., diazepam). As many BZs are extensively metabolized by the cytochrome P450 system in the gastrointestinal tract and liver and, furthermore, many of the metabolites are pharmacologically active, the duration of action of a BZ administration is often unrelated to the biotransformation of the parental compound. Two extreme examples are diazepam and flurazepam with their main and bioactive metabolites *N*-desmethyl-diazepam (nordiazepam) and *N*-desalkyl-flurazepam, respectively, which are eliminated from plasma with half-lives of 80–100 and 50 h, respectively. These differences readily explain that ultra-short-acting ligands are more suitable for the induction of sleep, longer acting ones suited for sleep maintenance and sedation, and very long acting BZ ligands for long-term treatment of anxiety, respectively.

All BZ compounds with nonfused substitutions at position 1 or 2 of the seven-membered ring, like diazepam and flurazepam, undergo a rapid modification of this substituent, resulting in the active NOR metabolites (NOR = *nitrogen ohne radikal*, German, meaning a nitrogen without any substituent). Thus, given the structural similarity of the BZ it is not surprising that quite a number of metabolic pathways converge on nordiazepam (Fig. 3.1). This compound can be metabolized to the active oxazepam by hydroxylation at position 3 of the diazepine ring before it is finally inactivated through glucuronidization at this hydroxyl position and excreted. As for compounds with a fused ring between positions 1 and 2 (midazolam, triazolam, and alprazolam; Fig. 3.2), the first step in inactivation cannot be performed and they are directly hydroxylated, glucuronidized, and excreted.

Non-BZ compounds such as the hypnotics zaleplon, zopiclone, and zolpidem (Fig. 3.4) are generally rapidly absorbed and reach their maximal concentrations within 1–2 h. Zopiclone is extensively metabolized by N demethylation, N oxidation, and decarboxylation. Its chiral center leads to stereoselective pharmacokinetics. Zolpidem is oxidized in the methyl groups and hydroxylated in the imidazolepyridine ring system [89].

Cytochrome P450 (CYP) 3A4 oxidase, located in the enterocytes of the small bowel and liver hepatocytes, is involved in the metabolism of a number of BZ receptor ligands, especially midazolam, triazolam, alprazolam, diazepam, flunitrazepam, and zopiclone [90–92]. The enzyme is inhibited by a number of other drugs, including the antidepressants fluoxetine and nefazodone, the macrolide antibiotic erythromycin, antifungal azoles, and the human immunodeficiency virus (HIV) protease inhibitor ritonavir as well as grapefruit juice. In the context of the mentioned BZ receptor ligands inhibition of CYP3A4 may lead to prolonged sedation, especially when patients are under stable drug treatment and one of the inhibitors is added. For example, midazolam plasma levels (i.e., areas under the curve) at the same dose can vary 400-fold depending on whether CYP 3A4 inhibitor (e.g., itraconazole) or inducer (e.g., rifampicin) is being coadministered [93], which produces clear effects on its clinically wanted sedative and unwanted motor-impairing actions.

3.3 GABA_A/BENZODIAZEPINE RECEPTORS

3.3.1 Subunit and Subtype Structural Diversity

GABA_A receptors are members of the superfamily of ligand-gated ion channels which should be termed Cys loop receptors in order to exclude the structurally unrelated ionotropic glutamate receptors. They are heteropentameric ion channels, although the initial purification of bovine GABA_A receptors suggested that only two proteins are involved in the formation of these Cys loop receptors [10]. Now it is common knowledge that GABA_A receptors are composed of an array of polysubunits (α_{1-6} , β_{1-3} , γ_{1-3} , ϵ , δ , π , and θ), all of which are products of separate genes [94–96]. Their variety is even intensified by several splice forms, for example, α_6 , β_2 , and γ_2 subunits.

The ρ_{1-3} subunits mainly or exclusively present in the retina show similar structural characteristics and exhibit a high sequence identity to the above-mentioned subunits. They are picrotoxin sensitive but insensitive to bicuculline, the prototypic competitive GABA_A antagonist, and to baclofen, the prototypic GABA_B receptor agonist, and have thus been classified as GABA_C receptor subunits [97, 98]. The classification of GABA_A and GABA_C receptors basing on a single pharmacological characteristic is controversial as only few features are common to all GABA_A receptors. Thus, Kai Kaila proposed the terms GABA_i and GABA_m for GABA_A + GABA_C and GABA_B receptors, respectively, with *i* standing for ionotropic and *m* for metabotropic (personal communication to HL).

Common features of all these subunits as well as for the glycine and nicotinic acetylcholine receptor subunits include four putative transmembrane regions and the so-called cysteine loop, located in the N-terminal extracellular domain and characterized by two cysteine residues spaced by 13 otherwise largely divergent amino acids which gave the group its name. Another hallmark of GABA_A receptors is a conserved sequence in the second transmembrane region encompassing the amino acids TTVLTMTT. Though this sequence has been used to retrieve 13 of the known GABA_A receptors [99], it is only partially conserved in the more recently identified subunits ϵ , π , and ρ_{1-3} and hardly recognizable in θ . In view of the fact that five of the eight amino acids are possibly lining the ion channel proper [100], the divergence of the ϵ and θ subunits warrants further investigation into the channel properties of receptors containing these subunits. However, with respect to BZ neither the ϵ nor the θ or δ subunits confer sensitivity to these ligands and the resulting receptors have not been shown to respond to these ligands. All subunits contain recognition sites for N glycosylation in the so-called large intracellular loop between transmembrane regions 3 and 4. The glycosylation adds to the theoretical molecular weight of the subunits, which ranges from 52 kDa for the unprocessed α_1 subunit, that is, still containing the 20- to 30-amino-acid-long leader sequence necessary for plasma membrane targeting, and 62 kDa for the α_4 subunit. This varies only marginally between mammalian species (e.g., between man, mouse, or rat), as the sequence identity between these species is mostly higher than 98%. Furthermore, no substantial pharmacological differences have been observed between the mentioned species. Exceptions to the rule are the non-BZ-relevant ϵ and θ subunits, which display only about 70% sequence identity between human, mouse, and rat [96].

BZ receptor-positive modulator ligands, such as diazepam, CL 218,872, and zolpidem, distinguish two GABA_A receptor subtypes differing mainly in their α - and

γ -subunit variants [101]. They characteristically display a high affinity to the α_1 -subunit-containing receptors, but CL 218,872 and zolpidem differ from diazepam in having reduced affinity to α_2 -, α_3 -, and α_5 -containing receptors [102, 103]. All these ligands are inactive at α_4 - and α_6 -subunit-containing receptors. This classification can be extended further, since some $\alpha\beta\gamma$ combinations differentiate between these ligands: zolpidem binds with poor affinity to α_5 - and/or γ_3 -subunit-containing receptors [104], while CL 218,872 has 10-fold higher affinity (low nanomolar) toward $\alpha_1\beta_3\gamma_3$ receptors than to any other α_1 - or $\gamma_{2/3}$ -subunit-containing receptors. The functional significance of this interaction has not been studied, but if existing at all in native brain, it represents only a minor pool of receptors as CL 218,872 fails to distinguish any high-affinity components in displacement analysis with rat hippocampal and cerebrocortical receptors [105]. This is but one example of the special properties that can be observed in recombinant receptors but does not seem to exist in appreciable amounts in native brain. It is important to note that the behavioral profiles of diazepam, CL 218,872, and zolpidem are quite different and their behavioral efficacy cannot be deduced from competitive ligand binding assays. Thus, CL 218,872 is a low-efficacy anxiolytic, diazepam a potent anxiolytic, and zolpidem a very potent hypnotic with little anxiolytic efficacy. These differences may be explained by their efficacies: CL 218,872 is a partial positive modulator, diazepam a wide-range full-partial positive modulator, and zolpidem an α_1 -subunit-preferring full positive modulator [106]. Therefore, competitive binding assay results need to be complemented with data on the intrinsic efficacies of the compounds before making any behaviorally relevant predictions. Even in the case that both data sets are available, behavioral effects may be due to receptor populations that cannot be mimicked in vitro. This has been seen, for example, in the granule cells of the cerebellum: Though the array of subunits is restricted to α_1 , α_6 , β_2 , β_3 , and δ , only 50% of the pharmacology seen in rat brain slices could be accounted for by any of possible subunit combinations in vitro [107]. The same issue becomes important when one attempts to apply in vitro selectivity data into human brain imaging studies with the purpose of visualizing various subtypes of GABA_A receptor.

Exchanging the β subunit in ternary $\alpha_i\beta_f\gamma_2$ receptors did not significantly alter the BZ binding characteristics [108, 109] for flunitrazepam, DMCM, FG8205, zolpidem, or CL 218,872 between the β_1 , β_2 , and β_3 isoforms in electrophysiological recordings [109]. Accordingly, Puia [110] reported for diazepam or bretazenil only a tendency toward a decreased potentiation while exchanging β_1 with the β_2 or the β_3 subunit in $\alpha_i\beta_f\gamma_2$ receptors. Sigel, however, observed a severalfold higher potentiation in $\alpha_{1/3}\beta_2\gamma_2$ receptors, as compared to $\alpha_{1/3/5}\beta_1\gamma_2$ [111]. The minor relevance of the β subunits in ternary receptors to BZ pharmacology was also seen when studied by [³⁵S]TBPS binding, although, in $\alpha_5\beta_f\gamma_{2/3}$ and to a lesser extent in $\alpha_3\beta_f\gamma_{2/3}$ receptors, the β_3 variant was required for high-affinity [³⁵S]TBPS binding [101, 104]. Interestingly, this correlates with the notion that α_5 -subunit mRNA colocalizes with β_3 mRNA [112, 113]. Another study on homo-oligomeric β_3 channels reported this subunit to be sufficient for high-affinity [³⁵S]TBPS binding [114].

The receptor structures are further altered by posttranslational modifications, but the roles of these processes in function and in pharmacological specificity have not been well established. The receptor subunits show sequence similarity of about 70% within classes and about 30% between classes.

3.3.2 Functional Domains

3.3.2.1 GABA and BZ Binding Pocket. Addition of the neurotransmitter triggers a small rotation of the extracellular domains of the receptor subunits [115], which then opens the channel pore formed by the adjoining transmembrane 2 TM2 regions of the five subunits as predicted from the data obtained with nicotinic acetylcholine receptors [116]. Using disulfide bond mapping in recombinant GABA_A $\alpha_1\beta_1$ mutant receptors, Horenstein et al. [117] demonstrated that the extracellular portion of the TM2-lined pore is more flexible than the intracellular portion and that these domains of the α_1 and β_1 subunits rotate asymmetrically, since homologous residues (α_1 T261C and β_1 T256C) form disulfide bonds only when the receptors are activated by GABA. The resulting covalent modification keeps the channels open.

The physical pore properties of GABA_A receptors are remarkably invariant among different subunit compositions (see [118]). Still, different compounds exert their action on the receptor via a range of different modes. For example, pentobarbital increases the mean duration of opening time and the mean number of openings per burst [119], but, as described before, BZ-positive modulators increase the open frequency [119] and BZ-negative modulators decrease it. Furthermore, though picrotoxinin does not directly interact with the binding site for pentobarbital, it produces the opposite effects on the receptor; that is, it reduces the mean number of openings per burst and shortens the mean open time [119]. Picrotoxinin protects the covalent modification of an α_1 V257C substitution by a sulfhydryl reagent in the intracellular portion of the TM2 region [120], possibly because of a direct steric hindrance by picrotoxinin, suggesting the direct blockade of the channel pore by ligands of this type.

The minimal structural requirement for GABA_A receptors gated by GABA is a heteropentamer built from two different subunits with one peptide derived from the α class and the other from the β class of variants [121]. Thus, it was expected that both subunit classes contribute to the formation of the GABA binding pocket. Indeed, a number of amino acids on members of both classes, most of them conserved within a subunit class, have been identified as being involved in high-affinity agonist binding. The first one in this series was recognized by the F-to-L mutation at position 64 in the rat α_1 subunit in an electrophysiological assay [111, 122], later confirmed by direct photolabeling of the site with [³H]muscimol [123]. Whereas the homologous residue in the α_5 variant was shown to be involved in the formation of the GABA binding pocket, the equivalent residues in the β_2 and γ_2 subunits do not affect GABA binding [122]. The two neighboring amino acids R66, corresponding to R70 in α_5 , and S68 in the α_1 variant have been reported to contribute to the GABA binding domain [124, 125], as well as R120 in α_1 and its counterpart R123 in α_5 [125, 126].

[³H]RO 15-4513 (Fig. 3.3) binding to BZ sites is differently modulated by GABA in various recombinant receptors. In $\alpha_1\beta_2\gamma_2$ receptors, GABA reduces its binding, which is in agreement with the classification of RO 15-4513 as a negative modulator [127]. In the $\alpha_6\beta_2\gamma_2$ receptors, GABA enhances significantly the binding. [³H]RO 15-4513 binding is sensitive to diazepam in $\alpha_1\beta_2\gamma_2$ ($K_i = 16$ nM [128]) and $\alpha_6(Q100)\beta_2\gamma_2$ ($K_i = 1.3$ μ M [129]) receptors but insensitive in $\alpha_6\beta_2\gamma_2$ receptors. These actions are also consistent with electrophysiological results ([130, 131], but see [132]), demonstrating that negative modulators act like positive modulators at $\alpha_6\beta_x\gamma_2$ and $\alpha_4\beta_x\gamma_2$ receptors. The observation that the intrinsic activities of flumazenil, ranging from

antagonistic to partial positive modulatory, and RO 15-4513, ranging from partial negative modulatory to partial positive modulatory, depended on the amino acid replacing the H101 [133] supports the idea that the amino acid at this position affects the intramolecular transduction of an allosteric effect and not only the ligand binding domain structure.

In the years after cloning the first GABA_A receptor subunit complementary DNAs (cDNAs) site-directed mutagenesis has contributed to the identification of residues on single subunits in a receptor complex involved in specificity, selectivity, and efficacy differences of BZ receptor ligands. In many cases the approach proceeded over the construction of chimeric proteins derived from two subunits with largely differing properties to finally point to a single amino acid. Another possible procedure employed the high sequence identity between subunits of a given class to directly point to amino acid candidates explaining physiological and pharmacological differences between GABA_A receptors. The first approach was used to largely explain the molecular basis of the so-called BZ type I and type II receptors. An E is conserved in all α variants at the position 201 besides in α_1 . Its exchange by a G leads to an increase in the affinity for the α_1 -preferring compounds CL 218,872 and 2-oxo-quazepam [134]. Further identified amino acids are T208 and I215 of the human α_5 subunit which confer high subtype selectivity of the partial inverse agonist L-655,708 to $\alpha_5\beta\gamma_2$ receptors in α_5 (T208S, I215V) $\beta_1\gamma_2$ receptors [135], the homologous residues affecting also the affinity of zolpidem in α_1 (S208T, V215I) $\beta_1\gamma_2$ receptors. However, these residues only slightly reduced the affinity of CL 218,872 binding. Still, these effects stress the importance of the whole domain between amino acid residues 201 and 215 in BZ binding [136, 137].

One of the most instrumental amino acid residues in the elucidation of BZ pharmacology is the H (in α_1 , α_2 , α_3 , and α_5) to R (in α_4 and α_6) transition at a position corresponding to R100 in α_6 . The single R-to-H substitution at positions 99 of α_4 and 100 of α_6 imparts sensitivity of these receptors to diazepam [137, 138]. Furthermore, diazepam insensitivity can be conferred to α_1 receptors by replacing the corresponding H101 with an R [138]. The point mutation only changes the affinity for diazepam but does not interfere with the affinity for GABA [139], a fact that has been utilized to dissect the behavioral pharmacological actions of classical BZs such as diazepam and at least partially attribute their diverse actions on receptors containing individual α subunits (see below). As well, diazepam-insensitive $\alpha_6\beta_2\gamma_2$ receptors can be converted to a diazepam-preferring species by four amino acid exchanges in the α_6 variant, thus reversing the rank order of potency of BZ receptor ligands in mutated as compared to wild-type receptors [137].

As briefly noted before, the same amino acid residue in the α_6 subunit has been identified as being polymorphic in rats: Alcohol-insensitive (AT) and alcohol-sensitive (ANT) rat lines were developed by selective outbreeding for differential sensitivity to the motor-impairing effects of an acutely administered moderate dose of ethanol (2 g/kg) [140]. The motor impairment was measured with a tilting plane test on a rough surface [141], which evaluates quick postural adaptations, supposedly needed cerebellar adjustment. The ANT rats are abnormally BZ agonist sensitive and have also slightly greater sensitivity to the motor-impairing actions of barbiturates, intravenous anesthetics, *N*-methyl-D-aspartate receptor antagonists, and neurosteroid agonists [142–145]. There are no overall differences in brain GABA_A receptors between the ANT and AT rat lines [146, 147], but the binding of [³H]RO 15-4513 to

the cerebellum in ANT rats is about 100-fold more sensitive to diazepam and lorazepam than the binding in AT rat samples [148, 149], resulting in reduced “diazepam-insensitive” BZ binding in ANT rats. This is caused by a single nucleotide exchange in the GABA_A receptor granule cell-specific α_6 subunit gene in the ANT rats leading to an R-to-Q exchange [150]. Interestingly, even if ANT rats differ from AT rats only in their cerebellar granule cell sensitivity to diazepam, in behavioral tests for the anxiolytic activity of diazepam they show heightened responses as compared to AT rats [151], suggesting that the cerebellum is also important for emotional behavior. Interestingly, the highly alcohol sensitive ANT rats consume voluntarily less alcohol than the alcohol-insensitive AT rats [152]. In proof, Saba et al. [153] have recently described a Sardinian alcohol-nonpreferring rat line to spontaneously have exactly the same mutation as the ANT rats in the α_6 subunit. However, whether these presumably α_6 -subunit-mediated effects of alcohol are due to receptors containing the γ_2 subunit and have to be thus classified as GABA_A/BZ receptors have still to be evaluated, especially in light of the more recent publication of the high sensitivity of $\alpha_6\beta_3\delta$ receptors against ethanol [154].

As outlined before, all GABA_A receptors of the composition $\alpha_i\beta_j\gamma_k$ ($i = 1, \dots, 6$, $j = 1, \dots, 3$, $k = 2, 3$) are BZ sensitive. Thus, it was only a question of time before the γ_2 subunit was molecularly dissected to identify amino acids crucial in BZ pharmacology. In this line, a single amino acid residue has been identified in the γ_2 subunit, which seems to critically determine the efficacy of a given BZ receptor ligand with a GABA_A receptor subtype [155]. If T142 in γ_2 is converted to serine, the channel response of the resulting $\alpha_1\beta_1\gamma_2$ receptor to 5 μ M GABA is increased by flumazenil and RO 15-4513 (instead of being unaffected or decreased in the corresponding wild-type receptors), thus converting negative or neutral allosteric modulators into positive ones. The F at position 77 of the γ_2 subunit is homologous to the above-mentioned F64 in the α_1 and α_6 subunits. Whereas the latter is required for high-affinity GABA functionality, the former is involved in high-affinity binding of several BZ receptor ligands, for example, zolpidem, the β -carboline DMCM, diazepam, and CI 218,872 [136, 156]. A similar correspondence between GABA and BZ responsiveness was found for amino acids involved in the GABA recognition on the β subunit [157] and BZ binding site on the homologous α -subunit residues [158]. Both findings substantiate the claim that the BZ binding site is a “converted” agonist recognition site.

Recently, two domains in the γ_2 subunit have been identified as transducing elements for the BZ activity, one being in the region TM1, the other in the adjacent region TM2 including the following short extracellular loop [159]. Together these domains may form the structural basis for the enhancement of GABA-induced currents by BZ receptor ligands.

For some BZ site ligands, such as β -carbolines, an additional binding site on GABA_A receptors independent of γ_2 subunits has been suggested [160, 161]. In addition to the function as a negative modulator on the BZ site at low micromolar concentrations, DMCM, ethyl- β -carboline-3-carboxylate (β -CCE), and propyl- β -carboline-3-carboxylate (β -CCP) at high micromolar concentrations potentiate the GABA_A receptor function through a supposedly loreclezole-associated binding site in the β_2 and β_3 subunits [162]. This positive modulatory effect is independent of the α variant present in the receptor complex and is more pronounced in α_6 -containing receptors due to the lack of inhibition (negative modulation) by the BZ binding site

[160, 161]. This site can be detected especially in the cerebellar granule cell layer by using [^{35}S]TBPS autoradiography, and it is decreased in the absence of α_6 subunits [163] but retained in $\gamma_2(\text{F77I})$ point-mutated mice [164], which show absence of DMCM-induced convulsions and presence of DMCM-induced motor impairment. As the effects are insensitive to flumazenil, they cannot be classified as being BZ receptor mediated though they are due to long known BZ receptor ligands.

An additional low-affinity BZ site has been suggested based on receptor assays in frog oocytes [165]. Diazepam, flunitrazepam, and midazolam but not flurazepam at micromolar concentrations produced strong enhancement of currents induced by a low GABA effective concentration (EC_3 sic!) in $\alpha_1\beta_2\gamma_2$ as well as $\alpha_1\beta_2$ receptors. In line with the subunit restrictions given before, nanomolar concentrations of all tested BZ agonists enhanced GABA responses in $\alpha_1\beta_2\gamma_2$ receptors, but to a lower extent than the micromolar concentrations of the active ones in γ_2 -less receptors. Importantly, only the nanomolar actions were blocked by the selective antagonist flumazenil. It remains to be shown in vivo whether these effects have any functional role, the simple test being the demonstration of flumazenil-insensitive sedation or anesthesia by the above-mentioned active BZs.

3.3.2.2 Assembly, Clustering, and Surface Expression. Most but not all GABA_A receptors assemble as *heteropentamers* and require signaling sequences for the specific interaction of the subunits. One of the sequences was identified employing a natural splice variant of the α_6 subunit which is alternatively spliced in about 20% of its transcripts in rat brain, causing a 10-amino-acid deletion of the amino acids E57 up to Q66, thus including the residues F and R, positions 63/65 and 64/66 in α_6 and α_1 , respectively [166]. When this spliced α_6 subunit is expressed in HEK 293 cells together with β_2 and γ_2 subunits, no binding activity of GABA or any BZ ligand is detected. Similarly, when the same subunits are expressed in *Xenopus* oocytes, no GABA-responsive channels are formed, though the transcript is translated in vitro. On a first glance, this result corroborates the idea that the GABA and/or BZ binding domains are at least partly in the extracellular region of the α subunits. Taylor et al. [167] have, however, shown that the short alternatively spliced α_6 subunit does not assemble into receptors that reach the plasma membrane, indicating that the deleted domain or a subsequent tertiary structural alteration affects membrane targeting. A stretch of 70 amino acids in the second half of the N-terminal extracellular domain was identified to be important for the homo-oligomeric assembly of the GABA_C receptor ρ_1 but not the ρ_2 subunits [168]. In rat α_1 and γ_2 subunits, domains have been detected [$\alpha_1(80-100)$ and $\gamma_2(91-104)$] that are necessary for subunit interaction, assembly, and formation of BZ binding site [169, 170] in recombinant $\alpha_1\beta_3\gamma_2$ receptors. Another adjacent region of the γ_2 subunit [$\gamma_2(83-93)$] might be needed for interaction with β_3 subunits [170].

Recently, a glia-derived protein was identified in the CNS of the mollusk *Lymnaea* [171]. This protein binds acetylcholine, shows a 15% sequence identity to the N-termini of nicotinic acetylcholine receptor subunits at domains that are suggested to be important in the formation of the agonist binding sites, and contains a cysteine loop with 12 (instead of 13) intervening amino acids. It lacks the membrane-spanning domains, thus forming soluble (i.e., non-membrane-bound), *homopentameric* complexes [172]. This stresses the importance of the extracellular N-terminus for the assembly of subunits in this family of ligand-gated ion channels. Importantly, there

are already data to suggest that at least BZ binding sites can be formed by truncated N-terminal extracellular domains in GABA_A receptor α_1 - and γ_2 -subunit dimers, whereas [³H]muscimol binding apparently requires also transmembrane domains of the α_1 subunits together with truncated β_3 subunits [170].

The altered pharmacology of native GABA_A receptors and changes in the γ_2 - and α_4 -subunit levels in δ -subunit-deficient mice indicate that the δ subunit preferentially assembles in the forebrain with α_4 subunits, where it interferes with the coassembly of α_4 and γ_2 subunits, the γ_2 subunit being recruited into additional functional receptors in its absence. This provokes the question of how GABA_A receptor subunit assembly is regulated in the normal brain. Little further is known besides that the N-terminal domains of the α subunits are obligatory for this process [167, 173], elegantly proven by the assembly of the homopentameric acetylcholine binding protein of *Lymnaea* [172]. Two scenarios exist to explain these results, that is, either the concentration of δ subunit exceeds that of the γ_2 subunit or the δ subunit has a higher probability than the γ_2 subunit in assembling with α_4 and α_6 subunits. In both scenarios, δ and γ_2 subunits compete with each other during assembly into functional receptors in neurons, a process which could efficiently limit the number of receptor subtypes, that is, subunit combinations, produced and especially the number of BZ-responsive GABA_A receptors. However, recombinant $\alpha_4\beta_3\gamma_2\delta$ GABA_A receptors are formed in HEK 293 cells [174] where the γ_2 and δ subunits assemble into functional receptors as demonstrated by their selective electrophysiological and pharmacological properties, though at a reduced expression level. However, fibroblasts may lack molecular features such as clustering proteins [175–177] that might be needed for the selective assembly or ideal function of subunits in vivo, thus further enlarging the once-thought narrow gap between the in vitro and in vivo properties of GABA_A receptors.

The γ_2 and γ_3 subunits not only confer BZ sensitivity to the resulting GABA_A receptors but also are instrumental in the subcellular targeting of the receptors, that is, the clustering of the receptors and/or the synaptic versus the extrasynaptic location of the receptors. Two main players have been identified to be involved in these cellular processes, gephyrin and GABA_A receptor-associated protein (GABARAP). In the following we will briefly outline their properties. Gephyrin, initially described as a 93-kDa protein copurified with glycine receptors [178], is now known to be more widely expressed in the CNS as well as peripheral tissue, even in areas devoid of the glycine receptor [179]. Mice lacking gephyrin die at day 1 after birth (P1) and exhibit a reduced number of clustered glycine receptors at their synapses but not an overall loss of glycine receptors [180]. As well, a significant reduction in the punctuate immunoreactivity toward the GABA_A receptor α_2 and γ_2 subunits is observed in spinal cord sections of these mice [181]. In primary hippocampal neuronal cultures synaptically clustered GABA_A receptors are reduced but their intracellular pool is increased. Together with results from γ_2 knockout mice, which exhibit a loss of clustered GABA_A receptors [182, 183], these data provide evidence for a dominant role for gephyrin and probably the $\gamma_{2/3}$ subunits in GABA_A/BZ receptor clustering. The pool of GABA_A receptors clustered extrasynaptically, as detected by $\beta_{2/3}$ - and γ_2 -specific antibodies in wild-type hippocampal neurons, is reduced during development, but even after 30 days in culture it amounts to 50% of all clusters [184]. This leaves open the question on the mode of specific targeting of these clusters or the precise subunit composition of extrasynaptic versus synaptic clusters.

Employing the yeast two-hybrid system, a ubiquitously expressed protein was identified to interact with a part of the large intracellular loop of the γ_2 subunit [185]. In spite of its distribution pattern it was called GABARAP. It exhibits sequence similarity with light chain 3 of microtubule-associated proteins and a putative tubulin binding motif, which apparently directly interact with microtubules and tubulin, respectively [186]. Recombinant $\alpha_1\beta_2\gamma_{2L}$ receptors expressed together with GABARAP have variable GABA sensitivity and channel kinetics depending on whether the receptors are in clusters or diffusely distributed on the cell membrane [175]. Using again the technique of yeast two-hybrid screening, Kanematsu et al. [187] found an inositol 1,4,5-trisphosphate binding protein, called p130, that may bind to GABARAP and inhibit the binding of γ_2 subunit to GABARAP. The p130 knockout mice show reduced sensitivity to diazepam in both behavioral and hippocampal electrophysiological experiments, whereas GABA-induced receptor currents are unaltered. The roles of the GABARAP and p130 are still unresolved, especially as the GABARAP-deficient mouse line appears to have no defects in GABA_A receptor membrane targeting [188].

Insertion of proteins into the plasma membrane and the half time of surface expression represent a highly regulated process. Thus, it is not really astonishing that there are proteins regulating these processes. In this line the ubiquitin-like protein Plc-1 has been found to interact with several α and β subunits of the GABA_A receptor [189]. This protein seems to be important for facilitation of GABA_A receptor surface expression and intracellular stabilization of subunits. Recently, it was shown that GABA_A receptors are constitutively internalized by clathrin-dependent endocytosis [190], which could be traced to the interaction of β and γ_2 subunits with the adaptin complex AP2. This interaction may be functionally important in vivo as the blockade of endocytosis increased the amplitude of GABA-induced miniature inhibitory postsynaptic currents in hippocampal neurons by two. The internalization of the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors in HEK 293 cells is strongly modulated by phosphorylation/dephosphorylation reactions [191], but the modulations might not be GABA_A receptor specific. Especially, the role of protein kinase C-mediated phosphorylation of receptor interacting proteins needs to be assessed. The functional and pharmacological regulation of receptor surface expression, subunit stabilization, and internalization thus remain to be studied, but the present scarce data already suggest that these mechanisms may also vary between different GABA_A receptor subtypes [192].

Whether the factors described here contribute to the mentioned discrepancy between the observed in vitro and in vivo effects of BZ receptor ligands will be a major focus of BZ research. Further, they might be important for the receptor regulation during tolerance development. Even more so, the results may even point to future pharmacological targets to treat disorders currently medicated with BZ receptor ligands.

3.3.3 Diversity of Brain Distribution

Early autoradiographic experiments have shown GABA_A receptor-associated GABA site labeling using [³H]muscimol as a ligand [193]. More thorough examination, however, has raised the suspicion that the high-affinity binding of GABA site ligands does not distribute as widely in the CNS as BZ site or channel site ligands labeled by [³H]BZ and [³⁵S]TBPS, respectively. The GABA binding site as seen with [³H]muscimol is concentrated especially to the cerebellar granule cell layer and thalamus, whereas the diencephalon and colliculi are hardly labeled contrasting with

the labeling patterns by [³H]BZ and [³⁵S]TBPS. Thus, [³H]muscimol autoradiographies indicate that GABA site labeling reveals only a fraction of all GABA_A receptors, though it is the prototypic agonist acting on all GABA_A receptor subtypes in functional assays at high nanomolar–low micromolar concentrations. High-affinity [³H]muscimol binding to rat brain sections was proposed to be associated with δ -containing receptors [194, 195]. This was corroborated by the findings that [³H]muscimol binding to GABA sites is reduced in cerebellar sections from α_6 knockout mice [163] exhibiting a reduced number of α_6 - and/or δ -subunit-containing receptors [196] and that in δ -deficient mice [197] the high-affinity [³H]muscimol binding is reduced in both the cerebellum and forebrain [145]. In contrast, [³H]RO 15-4513 binding to BZ sites of the cerebellum and forebrain is increased in $\delta^{-/-}$ animals, which was, at least partly, due to an increase in diazepam-insensitive receptors. Concurrently the amount of the γ_2 subunit, as determined by Western blotting, is increased and that of α_4 decreased in $\delta^{-/-}$ animals, while the level of the α_1 subunit remains unchanged [145], indicating augmented assembly of γ_2 subunits with α_6 and α_4 subunits in the absence of the δ subunit. This points to an increased expression of BZ receptors as a compensation to the decrease of high-affinity GABA recognition sites [128, 198, 199].

As stated before, GABA_A receptors responsive to BZ receptor ligands require any α variant together with any β subunit and either γ_2 or γ_3 . In these configurations the β subunits are known to contribute little to the differences in the BZ ligand-mediated effects and the γ_3 subunit is rare (see below). Therefore, mainly the α subunits determine the pharmacological and physiological responses of GABA_A receptors to BZ receptor ligands. Thus, the following description focuses on the α_1 variant distribution in the CNS, especially as the γ_2 subunit is found in nearly all brain regions, though to different intensities [200].

The α_1 subunit appears to be the most abundant subunit in the CNS, missing only in a few regions and often colocalizing with the β_2 subunit [112, 113, 201–203]. Strong α_2 - and α_3 -subunit expression seems to inversely correlate with α_1 expression. These two subunits as well as α_3 and α_5 are abundant in the hippocampus [113, 202]. The $\alpha_3\beta_1\gamma_2$ -subunit combination is reported for serotonergic neurons of the raphe nuclei and cholinergic neurons of the basal forebrain [204–206]. Some subunits dominate during embryonic development, for example, α_2 , α_3 , and α_5 [202, 207, 208], but are reduced or even absent in defined regions in the adult brain [209]. The time-delimited presence of specific subunits during ontogenesis in defined brain regions appears to be essential for normal development [210, 211]. Other neurons, for example, the cerebellar Purkinje cells, maintain their subunit composition of $\alpha_1\beta_{2/3}\gamma_2$ throughout all pre- and postnatal stages [202]. It is also possible that more than one α subunit is in a pentameric complex [212, 213].

The α_5 subunit, concentrated in the adult hippocampus and olfactory bulb [113, 214], is part of a receptor with negligible affinity for BZ receptor ligands such as the imidazopyridine zolpidem [103, 215] (see Fig. 3.4). Major amounts of the α_4 subunit are found in the hippocampus and thalamus and often colocalize with the δ subunit [198]. The α_6 subunit, also found to colocalize with the δ subunit, appears to be almost exclusively restricted to the cerebellar granule cells [128] with some traces found in the dorsal cochlear nucleus [216, 217], a brain area developmentally derived from cerebellar precursors.

The β_1 mRNA signals are strongest in the hippocampus, less pronounced in the claustrum and parts of the stria terminale, and weak in the amygdala and

hypothalamus [113, 218, 219], whereas the β_2 subunit shows more widespread distribution that seems to be inversely correlated to the β_1 and β_3 concentrations, for example, in the hypothalamus and in parts of the hippocampus [113, 218, 219]. Here the β_3 subunit is strong, mainly in CA1 and CA2, but as well in the olfactory bulb, cortex, caudate putamen, nucleus accumbens, and hypothalamus. Like for the α variants, studies on the distribution of the β_1 , β_2 , and β_3 subunits during pre- and postnatal ontogeny indicate an independent regulation of their expression in different brain regions that suggests a role during development [202, 209, 220]. A line of proof is a mouse line devoid of the chromosomal region encoding α_5 and β_3 that bears a neonatally lethal cleft palate [221]. Introduction of a transgene coding for the β_3 subunit rescued these mice [211], thus proving not only the responsibility of β_3 for the cleft palate but also the role of the β_3 variant in development, in this specific case outside the CNS. These observations provoke the question of whether BZ can cause congenital malformations. Indeed, there have been early reports to this end [222, 223], which, however, could not be substantiated in more recent studies [224, 225].

The ubiquitous presence of the γ_2 subunit and hence the BZ receptor is contrasted by the restricted distribution of the γ_1 and γ_3 variants. Messenger RNA encoding the γ_1 subunit is limited to regions of the amygdala, septum, and hypothalamus [226]; the γ_3 subunit is largely present in the olfactory bulb, cortex, basal nuclei, and medial geniculate of the thalamus [99, 113, 227].

Immunoprecipitation studies with an α_6 antibody suggested that between 10 and 40% α_6 subunit is combined with the α_1 subunit [212, 228]. Caruncho and Costa [228a], however, concluded from double immunolabelings of freeze-fracture replicas that these two subunits do not colocalize within the same receptor complex. Based on mRNA and protein colocalization, further subunit combinations containing more than one α variant have been suggested, such as $\alpha_1\alpha_3\beta_{2/3}\gamma_2$ [203, 213, 229]. However, immunoprecipitation studies might include incompletely assembled receptors as well as receptors not inserted into the outer cell membrane [192]. As well some receptor pools might be underestimated in such studies because they are not readily solubilized by conventional detergent treatments [230].

In addition to the differences between [^3H]BZ and [^3H]muscimol binding distribution, there exists a large discrepancy between the high-affinity [^3H]GABA site ligand binding to brain sections, membrane homogenates, or recombinant receptor preparations on one side and the low-affinity binding of these ligands suggested from allosteric actions in functional assays employing electrophysiological methods and [^{35}S]TBPS and [^3H]BZ binding assays on the other side. Whereas the former is uniformly in the range of tens of nanomolar [104], the latter varies from high nanomolar to the micromolar range [101, 118, 231]. As this discrepancy holds true for single-receptor preparations, it cannot be due to receptor heterogeneity but rather must be an intrinsic receptor property. One likely explanation is the interconversion between a low-affinity and a high-affinity state, which, however, still awaits a definite biochemical proof.

3.3.4 BZ Functional Diversity as Revealed by Gene Knockout and Knockin Models

The most dramatic increase in knowledge about the functional diversity of BZ receptor ligands has been obtained in the past years by the use of knockout models and even more pronounced by mouse knockin models for GABA_A/BZ receptors.

These studies revealed that the deficiency of some subunits such as the GABA_A receptor γ_2 and the before-mentioned β_3 subunit cannot be compensated for in the developing brain. Mouse lines devoid of these subunits are severely compromised [210, 232], and thus absence of these subunits is incompatible with life, at least during development.

The α_5 -subunit gene, the expression of which is largely restricted to the hippocampus but as well present in parts of the cortex and more widespread in prenatal development, has been disrupted in mice and their behavior has been analyzed. They show enhanced platform learning in the Morris water maze and normal behavior and normal sensitivity to BZ agonists in the elevated-plus maze test of anxiety [233]. It is thus possible that inhibition of α_5 -subunit-containing receptors might help in improving cognitive functions, though the basic function of this subunit can obviously not be to curtail learning ability. A more philosophical aspect is the idea that forgetting is as essential for life as learning. Interestingly, as mentioned above, reduction of the expression of this subunit in the hippocampus seems to correlate with tolerance development to prolonged BZ treatment in animal models [75, 76].

The lack of γ_3 , α_6 , and δ subunits, and even in view of the data cited above, the lack of the α_5 variant does not produce any strong behavioral or physiological phenotype [197, 233–237]. Even more surprisingly, the homozygotic loss of α_1 or β_2 subunits resulting in a 50% reduction of all GABA_A receptors [238] is associated with neither lethal or strong behavioral effects, indicating functional compensation. However, though a general increase in other α -subunit expressions would be expected in these mice, the contrary was found: The α_6 -subunit-dependent BZ-positive modulator-insensitive [³H]RO 15-4513 binding is reduced by about 30%, suggesting that proper assembly or membrane targeting of the α_6 subunits needs the presence of α_1 subunits [238]. More recent experiments on the α_1 knockout mice have revealed clear compensations, especially by increased α_2 and α_3 subunits in the forebrain [239].

The proposal that the α_6 subunit plays a significant role in the action of alcohol [240] warranted an analysis of the behavior of α_6 -subunit-deficient mice behavior in response to sedative compounds [235, 236]. Two independently generated α_6 knockout lines lack the subunit-specific [³H]RO 15-4513 binding in the cerebellar granule cell layer but neither exhibit any immediately apparent motor learning or coordination deficits nor is their alcohol and anesthetic sensitivity different from that of wild-type animals [241]. However, their motor function is more readily affected by diazepam than that of the wild-type mice [242]. A potassium leak current carried via the Task-1 channel is increased in $\alpha_6^{-/-}$ mice, most likely needed to counteract the loss of inhibition in the cerebellar granule cells [243]. Analogous phenomena similar to this may occur in all other GABA_A knockout mice, obscuring the biological significance of the subunits and hindering the interpretation of the lack of apparent behavioral consequences.

To overcome these obstacles, Rudolph, Möhler, and co-workers as well as Rosahl, Whiting, and co-workers utilized a gene knockin approach that alters the diazepam-sensitive sites in α_1 -, α_2 -, α_3 -, and α_5 -subunit-containing receptors into insensitive ones by exchanging the H to R at the position N-terminal to the cysteine loop that is crucial for diazepam sensitivity [138]. These mouse models produced information on the behavioral roles of GABA_A receptor subtypes. Initially, this approach was thought to provide the final answers to the GABA_A receptor subtype-specific actions of BZ receptor ligands. In the first round of experiments two groups independently

reported that in α_1 (H101R) mutant mice diazepam is no longer sedative and amnestic, partially lost its anticonvulsant effects, but retained normal anxiolytic properties [244, 245]. However, α_1 (H101R) mice react normally to diazepam with respect to sleep-related parameters [246]; most like hypnotic sleep-related effects most likely involve other GABA_A receptor subtypes.

The α_2 (H101R) mice, that is, mice with a BZ-insensitive α_2 variant, did not display clear anxiolytic responses to diazepam, while their sedative responses to BZs were normal [247]. This is not surprising in view of the involvement of a number of brain regions in anxiety-related behavior, including the amygdala, a structure with high α_2 content, mammillary bodies, and dorsal hippocampus (see [113, 248]). More recent data, however, put into question the involvement of solely the α_2 subunit in the anxiolytic effects of BZ receptor ligands. Along this line, α_3 -containing receptors may mediate anxiolytic effects of novel subtype-selective BZ ligands [249, 250]. Interestingly, the α_3 (H126R) mice showed no deficits in the actions of diazepam [251], though the α_3 subunit is strategically expressed in important monoaminergic nuclei [203] believed to be involved in the regulation of emotional behavior. As well, even α_5 -containing receptors cannot be totally excluded to be involved in anxiety behavior [252]. Further experiments with these models will be important to exclude compensatory alterations in the GABAergic or other systems which may occur in spite of the absence of any proven endogenous BZ receptor ligands (see above) and in the absence of any overt physiological changes in GABA sensitivity of the mutated α variants. As well, the roles of these subtypes in behaviors such as tolerance and dependence to BZs need to be clarified. The muscle-relaxing effects of BZ receptor ligands seem to be largely mediated by the α_2 subunit as in the BZ-insensitive α_2 variant mice the myorelaxant action of diazepam was almost completely abolished [251].

As well, the role of the α_5 variant in the action of BZ receptor ligands did not become as clear as hoped for when mice carrying the H-to-R mutation were analyzed [253]: Diazepam no longer exerted the full muscle-relaxing effect seen with high doses of BZ ligands, but the sedative, anticonvulsant, and anxiolytic effects of diazepam were unaffected in these mutant mice. Surprisingly, the point mutation resulted in a selective reduction of α_5 -containing GABA_A receptors in hippocampal pyramidal cells resulting in an overall loss of 20% of the α_5 protein, which in itself did not change the above-mentioned effects but changed the response in a trace fear-conditioning task in which a tone associated with an electric shock was separated by an empty time interval independent of any external BZ. Though these results can be taken as a hint for the involvement of the α_5 subunit in some type(s) of associative learning, the role of BZ receptor ligands is still open for discussion. The same mutant mice did not develop any tolerance to the α_1 -mediated sedative effects of diazepam which was interpreted as a regulatory crosstalk between two GABA_A receptor subtypes [75].

3.4 STRUCTURE ACTIVITY RELATION OF BENZODIAZEPINES

In a strict sense all BZ receptor ligands are GABA_AR subtype selective as none of these ligands recognizes all native or heterologously expressed ligand-gated ion channels of this type, simply because, contrary to previous beliefs, not all GABA_AR

are BZ receptors. Slightly more relevant to the pharmacology of BZ receptor ligands is the observation that not a single ligand in clinical use binds to all BZ receptors with similar affinity, simply because no BZ receptor ligand recognizes GABA_A/BZ receptors containing the α_4 or α_6 subunits, resulting in the term “diazepam-insensitive” (DI) BZ receptors for $\alpha_{4/6}\beta_f\gamma_2$ receptors. However, these receptors bind the azido derivative of flumazenil, RO 15-4513, with high affinity that is identical to all receptors of the configuration $\alpha_i\beta_f\gamma_2$ ($i = 1, \dots, 4, 6$; $j = 1, 2, \dots, 3$) and this ligand binds to α_5 -containing BZ receptors with an even 10-fold higher affinity. Still, this selectivity does not play any role in clinical practice, whereas compounds which selectively recognize any given receptor of the types $\alpha_i\beta_f\gamma_2$ ($i = 1, 2, 3, 5$ with any $j = 1, 2, 3$) would be highly desirable.

Two different strategies have been employed to probe the structural requirements of BZ receptor ligands with specific receptor subtypes. One might be called the “molecular path,” in which putative subtype-selective compounds are tested on an array of recombinant receptor subunit combinations with biochemical and/or electrophysiological methods. The second one assumes that certain aspects of pharmacological BZ receptor ligand effects are mediated through defined receptor subpopulations and might be called the “behavioral path.” A third possible route has not yet been taken and presumably it will take some time before it will lead to a successful end in the field of the BZ receptor ligands. It takes advantage of structural data of the receptor to fit appropriate molecules directly into the pharmacophore. Especially the latter but also the first route is hampered by the continuum of effect size of these BZ receptor ligands from full negative modulators up to full positive modulators, though, on the other hand, this range of effect size has facilitated the development of so-called functional selective compounds, in which the given compound binds to some or all receptor subtypes but the efficacy is, at best, zero on one type and 100% on the other.

As far back as 1993, intrinsic efficacy differences were supported to mediate the differing effects of BZ receptor ligands [254], although the molecular diversity of GABA_A/BZ receptors was traced to the existence of several α variants already in 1989 [108]. A fuller appreciation to its now-known extent came in the following years [103, 128, 198], although BZ type I over BZ type II preferring compounds like CL 218,872, quazepam, and 2-oxo-quazepam were identified over 20 years ago [255–258]. The first subtype-specific structure–activity analysis was made under the assumption that in the cerebellum [³H]flunitrazepam binding represents a single receptor population [259], an assumption we now know to be essentially but not totally correct as only 90% of all diazepam-sensitive receptors in this brain region contain the α_1 variant and thus represent the BZ type I receptors. Zolpidem is now the most subtype-selective BZ receptor ligand in clinical use. It displays a 12- to 25-fold higher affinity to α_1 -containing receptors as to α_2 - and α_3 -containing receptors, paired with negligible affinity to all other α variant receptors [103, 260]. Recently, a zolpidem derivative was shown to be even more α_1 selective than zolpidem: This compound only recognizes the most abundant $\alpha_1\beta_f\gamma_2$ receptors [261] but still displays anxiolytic-like properties.

It took a few more years before the first molecular modeling approaches were performed in the search for α -subunit-preferring compounds with a single receptor population [262, 263], in this specific case, the α_6 -containing and thus one of the two theoretically possible diazepam-insensitive receptors. By analyzing close to 50 BZs

[263], the authors concluded that the ester substituent and substitutions at position 6 besides the obvious position 7 of imidazo-[1,4]benzodiazepines (compare Fig. 3.3) have a marked influence on the ratio of affinity for diazepam-sensitive to diazepam-insensitive receptor subtypes. However, no ligand was identified that had a substantially higher affinity to α_6 -containing receptors or a ratio of affinities substantially in favor of α_6 -containing receptors.

More comprehensive pharmacophore models were proposed in the following years and included not only the α_6 -containing receptors but also $\alpha_{1/2/3}$ - and especially α_5 -containing GABA_A/BZ receptors. For the latter it had been observed that imidazo-benzodiazepines are generally α_5 receptor selective [104, 264]. A lipophilic pocket present in the general pharmacophore of all GABA_A/BZ receptors [265] seems to be smaller in α_1 -containing receptors than that present in α_5 -containing receptors [264]. This may account for the high affinity of zolpidem to α_1 receptors as it may lead to a strong lipophilic-lipophilic interaction only possible in the restricted space. Thus, increasing the size of the substituent at position R7 in Figure 3.3 increased the selectivity for α_5 -containing receptors and finally resulted in the development of RY-80, a high-affinity, high-selectivity ligand for α_5 receptors [266]. A similar strategy yielded the α_5 -selective ligand L-655,708 that exhibits an affinity in the same order of magnitude [267]. By exchanging two amino acids in α_1 into the corresponding residues of α_5 (α_1 S205T,V212I) the binding selectivity of α_5 against L-655,708 was transferred to α_1 -containing receptors [135]. Both ligands as well as a number of other ligands identified as α_5 selective [268] are negative modulators at the BZ recognition site. A good candidate for a positive modulator on this receptor subtype is still missing, if at all desirable in view of the fact that negative modulators at this site are developed as putative "cognition enhancers," thus leading to the speculation that positive modulators should lead to learning deficits.

In recent years a number of compounds have been published that are selective for the other members beside α_5 of the former BZ type II receptor group, that is, α_2 and/ or α_3 receptors [250, 269]. However, in most instances the compounds are not selective for the latter receptors in the sense that they bind with a high enough difference in affinity to produce a subtype-selective effect but they more pronouncedly differ in their efficacies exerted on α_1 , α_2 , α_3 , and α_5 receptors. One of these compounds is a negative modulator and anxiogenic in vivo at doses that minimize the occupancy at other than α_3 receptor subtypes, again suggesting that the α_3 receptor subtype may play a role in anxiety.

3.5 FUTURE DEVELOPMENTS

The existing mechanisms to extrinsically regulate the BZ-modulated GABAergic system are widespread and of tremendous clinical importance, although they represent only a substructure of the GABA_A system, which again is only a part of all inhibitory processes in the CNS. Nonetheless, the entire clinical or scientific potential of the BZ-dependent inhibition has probably not been exhaustively explored, mainly due to the large heterogeneity of the GABA_A/BZ receptor system. We are still awaiting $\alpha_i\beta_x\gamma_2$ ($i = 2, \dots, 6$) receptor subtype-specific drugs to come into clinical practice. Even less is known of BZ receptors containing the γ_3 instead of the γ_2 variant. There is no reason to believe that a minor receptor population is of minor importance [270, 271]. As well, the old story of partial versus full modulators, at both

sides of the scale, that is, positive and negative modulators, has not completely unfolded [272]. It is most likely that the use of these “fine-tuning” substances could finally benefit patients. In the extreme, this could lead to a drug only suitable for the treatment of a minor population of affected persons, regardless of whether diagnosed with anxiety-related diseases, epilepsy, insomnia, or drug-related problems. Another open question is the differential activation of synaptic versus extrasynaptic receptors, though it is open to discussion whether this can be achieved at all with any BZ ligand.

As reviewed elsewhere, genetic variations of the GABA_A receptor subunit may affect the susceptibility to many neuropsychiatric diseases [88]. When we better understand the underlying, perhaps neuronal cell population-specific, pathophysiological mechanisms, we may be able to find a stronger rationale for developing improved BZ ligand-based therapeutics.

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4

NEUROACTIVE STEROIDS IN ANXIETY AND STRESS

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4.1	Introduction	134
4.2	Neuroactive Steroid Chemistry and Pharmacology	135
4.2.1	Brain and Peripheral Sources of Neuroactive Steroids	135
4.2.2	Actions on GABA _A Receptors and Other Ligand-Gated Ion Channels	137
4.2.3	Enantiomeric Selectivity of Neuroactive Steroids	141
4.2.4	Behavioral Effects	142
4.3	Effects of Neurosteroids in Animal Models of Anxiety and Stress	142
4.3.1	Elevated-Plus Maze	143
4.3.2	Geller–Seifter and Vogel Conflict Tests	145
4.3.3	Light–Dark Box	146
4.3.4	Acoustic Startle and Fear-Potentiated Startle	147
4.3.5	Mirrored Chamber	147
4.3.6	Open-Field Activity	148
4.3.7	Defensive Burying Behavior	149
4.3.8	Separation-Induced Ultrasonic Vocalizations	150
4.3.9	Modified Forced-Swim Test	150
4.3.10	Mild Mental Stress Models and Social Isolation	151
4.4	Neuroactive Steroid Interactions with Stress-Induced Behaviors	152
4.4.1	HPA Axis and Stress	153
4.4.2	Anxiety-Related Disorders	154
4.4.3	Depression	156
4.4.4	Acute and Chronic Effects of Alcohol	157
4.4.5	Stress-Induced Drug Reinstatement	159
4.5	Conclusions and Outlook	160
	Acknowledgments	161
	References	161

4.1 INTRODUCTION

Rapid membrane effects of certain specific metabolites of steroid hormones provide a mechanism by which these metabolites can influence brain function and behavior in addition to the classical genomic actions of the parent steroid hormones. It was over 60 years ago that the pioneering studies of Hans Selye [1] reported the sedative–anesthetic activity of the hormones progesterone and deoxycorticosterone, where among 75 steroids tested by systemic (i.p.) administration in rodents, 5 β -pregnane-3,20-dione was the most active. This led to the introduction of hydroxydione sodium (21-hydroxy-5 β -pregnane-3,20-dione succinate, sodium salt) as the first steroidal anesthetic in 1955 [2]. However, it was Margarethe Holzbauer and her colleagues, during the 1969–1985 period, who isolated and identified pregnenolone, progesterone, allopregnanolone (3 α ,5 α -THP), epiallopregnanolone (3 β ,5 α -THP), allopregnanedione (5 α -dihydroprogesterone, or 5 α -DHP), 20 α -dihydroprogesterone, and allopregnanediol (5 α -pregnane-3 α ,20 α -diol) from ovarian venous blood of the rat and measured the ovarian content and secretion rates of these steroids during proestrus [3]. Their work was seminal but is often forgotten. Holzbauer and colleagues [4] further demonstrated the *in vivo* secretion of pregnenolone, progesterone, and 3 α ,5 α -THP by the adrenal gland of the rat in quantities similar to those secreted by the ovary during estrus. Their laboratory later provided an outdated organic extract of adrenal venous blood that was found to contain similar amounts of allotetrahydrodeoxycorticosterone (3 α ,21-dihydroxy-5 α -pregnan-20-one; alloTHDOC) and 3 α ,5 α -THP by radioimmunoassay (RIA) after separation of the steroid fraction by high-performance liquid chromatography (HPLC; R. Purdy, unpublished).

Two decades ago, γ -aminobutyric acid A (GABA_A) receptors were found to be sensitive to modulation by steroids, providing the first hint at a mechanism underlying the reported rapid (on the order of seconds) onset of action of these steroids. Subsequent to the initial demonstration by Harrison and Simmonds [5] that alphaxalone (3 α -hydroxy-5 α -pregnan-11,20-dione, a metabolite of 11-dehydrocorticosterone found in patients with congenital adrenal hyperplasia exhibiting 21-hydroxylase deficiency [6]) potentiated GABA-gated chloride currents, evidence has accumulated that alphaxalone and structurally related steroid derivatives have rapid membrane actions via an interaction with ligand-gated ion channels [7–11]. This evidence has given rise to the term *neuroactive steroids*, to distinguish them from *neurosteroids*, which are synthesized *de novo* from cholesterol in nervous tissue [12]. Behaviorally, GABA agonist neuroactive steroids are now recognized to possess especially potent anesthetic, hypnotic, anxiolytic, and anticonvulsant properties [13].

An understanding of the physiological role of neuroactive steroids is essential for their acceptance as modulators of stress and anxiety. It is apparent that these endogenously occurring steroids can reach levels in the rat brain that are capable of modulating GABA_A receptors. 3 α ,5 α -THP and alloTHDOC have been measured in brain and plasma, where their levels have been shown to fluctuate in response to stress in rats [14], and during the estrous and menstrual cycles of rats and humans, respectively [8]. Brain and plasma levels of 3 α ,5 α -THP temporally follow those of progesterone in the female rat (i.e., peak brain levels are observed during estrus in the rat and dramatically increase during pregnancy). Both 3 α ,5 α -THP and alloTHDOC are detectable in the normal male rat brain, but the levels are low under most circumstances. However, exposure of male rats to stressors such as ambient

temperature swim, footshock, or CO₂ inhalation resulted in a rapid 4- to 20-fold increase in brain levels of 3 α ,5 α -THP and alloTHDOC [14, 15] to the equivalent of 10–30 nM. Similar concentrations are observed in female rats during estrus, while concentrations can increase to approximately 100 nM during pregnancy [8, 16, 17], documenting that basal 3 α ,5 α -THP levels are higher in females than in males. These concentrations achieved *in vivo* are within the range of concentrations previously shown to potentiate the *in vitro* action of GABA at GABA_A receptors, suggesting that fluctuations in endogenous 3 α ,5 α -THP levels in rodents and both 3 α ,5 α -THP and 3 α ,5 β -THP levels in humans may be physiologically relevant. Evidence in support of this idea has been reviewed recently [18].

4.2 NEUROACTIVE STEROID CHEMISTRY AND PHARMACOLOGY

4.2.1 Brain and Peripheral Sources of Neuroactive Steroids

The term *neurosteroid* was introduced by Baulieu [19] to designate a steroid hormone derivative found in brain at concentrations that were independent of its plasma concentration, and the story of their discovery and function has been recently reviewed [12]. Endogenous concentrations of the progesterone metabolite 3 α ,5 α -THP have been detected in brain, plasma, and adrenals of male and female rats and in female rat ovaries [4, 8, 15, 20–23]. Brain 3 α ,5 α -THP levels have been detected in rat, mouse, dog, monkey, and human [8, 15, 22–28]. Compared with 3 α ,5 α -THP, the 5 β -reduced epimer is present in the rat in much lower abundance, if at all [27]. In addition, brain 3 α ,5 α -THP concentration is detectable at lower levels in adrenalectomized animals and is higher than plasma 3 α ,5 α -THP levels in intact animals [15, 22, 23].

A number of studies have established that the enzymes identified in classic steroidogenic tissues are likewise found in the nervous system [29]. Thus, the endogenous concentration of neuroactive steroids in the brain most likely reflects a combination of neuroactive compounds produced there *de novo* as well as steroids metabolized to neuroactive compounds in the brain but derived from circulating precursors. For this reason, it has recently been proposed that the definition of the term neurosteroid be broadened to include both sources of neuroactive steroids [29]. It also is noteworthy that all neurosteroids identified to date have been found to have neuroactive effects in some behavioral assay. The most recent study identified the major neurosteroid in the amphibian brain as 7 α -hydroxypregnenolone and subsequently determined that this neurosteroid acts as a neuronal activator to stimulate locomotor activity of breeding newts [30]. A schematic of the biosynthetic pathway of neuroactive steroid formation from cholesterol is depicted in Fig. 4.1.

Many methods have been used to quantify steroidal compounds. These include RIA, gas chromatography–mass spectrometry (GC/MS), HPLC, and liquid chromatography–mass spectrometry (LC/MS). While these techniques are successful in the analysis of steroids, it has been difficult to achieve quantitative analysis of small samples of neurosteroids because of their low concentrations in nervous tissues. Highly specific analytical methods are required to analyze small quantities of neurosteroids and their sulfates. Only with extremely sensitive methods of analysis is it possible to discover whether neurosteroids are synthesized in nervous tissues in quantities sufficient to affect neuronal activity and whether these neurosteroids are distributed uniformly in brain.

In the initial identification of dehydroepiandrosterone sulfate (DHEAS) [31] and pregnenolone sulfate (PREGS) [32] in the rat brain, a conjugated steroid fraction from brain extracts was prepared by chromatography on a column of Sephadex LH-20 and termed the *sulfate fraction*. This was free from unconjugated steroids, steroidal esters of fatty acids (lipoidal steroids), and steroidal glucosiduronates. This sulfate fraction was then hydrolyzed by solvolysis in ethyl acetate for 12–16 h at 37°C, and the hydrolyzed products were purified on a column of Lipidex 5000. The purified steroids were converted to their trimethylsilyl ethers and characterized by GC/MS as DHEA and PREG. On the basis that the levels of DHEA and PREG separately measured from the hydrolyzed sulfate fraction from brain extracts by RIA were markedly elevated compared to corresponding levels in blood and were found in extracts of brain tissue from rats previously adrenalectomized and orchietomized, DHEAS and PREGS were described as neurosteroids. A considerable body of electrophysiological, pharmacological, and physiological work has subsequently been carried out on these two presumed neurosteroid sulfates [33]. Meanwhile, Prasad et al. [34], demonstrated that when extracts of brain were heated and treated with triethylamine or various reducing agents such as ferrous sulfate, larger amounts of DHEA and PREG could be measured by GC/MS, compared to simple extraction without such treatments. They suggested that there might be steroidal hydroperoxides or peroxides in brain that had not yet been characterized.

Recently, two laboratories have independently cast grave doubts on the existence of significant amounts of DHEAS and PREGS in the adult rat brain. Using a non-exchangeable internal standard of [$3\alpha,11,11\text{-}^2\text{H}_3$] allopregnanolone sulfate and a tracer amount of [$1,2,6,7\text{-}^3\text{H}_4$] DHEA sulfate, Liu et al. [35] were unable to find detectable amounts of DHEAS, PREGS, and any of the four pregnenolone sulfates in nonhydrolyzed extracts of the adult Sprague–Dawley male and female rats using LC/micro-electrospray MS (LC/micro-ESI–MS). Shimada et al. [36] also have found only low amounts, 0.53 ± 0.28 ng/g, of PREGS in nonhydrolyzed extracts from adult Wistar rat brain using a unique derivatization procedure followed by LC/micro-ESI–MS. This compares to the value of about 20 ng/g brain of PREGS originally reported using solvolysis and measurement by RIA [32]. At present, there are no reports of other PREG-containing compounds that could account for this 20-ng/g brain level. Thus, the nature of the majority of PREG-containing compound(s) in the sulfate fraction from extracts of the rodent brain remains a mystery. It would require low microgram amounts of such a compound to be identified (after purification) using the most sensitive microprocedure of high-resolution proton magnetic resonance instrumentation.

4.2.2 Actions on GABA_A Receptors and Other Ligand-Gated Ion Channels

After alphaxalone was found to potentiate GABA-gated chloride currents [5], the progesterone metabolite $3\alpha,5\alpha$ -THP and the deoxycorticosterone metabolite alloTHDOC were determined to be potent positive modulators of GABA_A receptors. The relationship between plasma levels of corticosterone and alloTHDOC, measured by RIA in various stages of immobilization stress in adult male Sprague–Dawley rats, is illustrated in Fig. 4.2. It also has been demonstrated that alloTHDOC can be formed in brain and in the periphery from $3\alpha,5\alpha$ -THP through 21-hydroxylase activity [37] of cytochrome P450 (CYP2D) isoforms (Fig. 4.1). Therefore, we do not know at

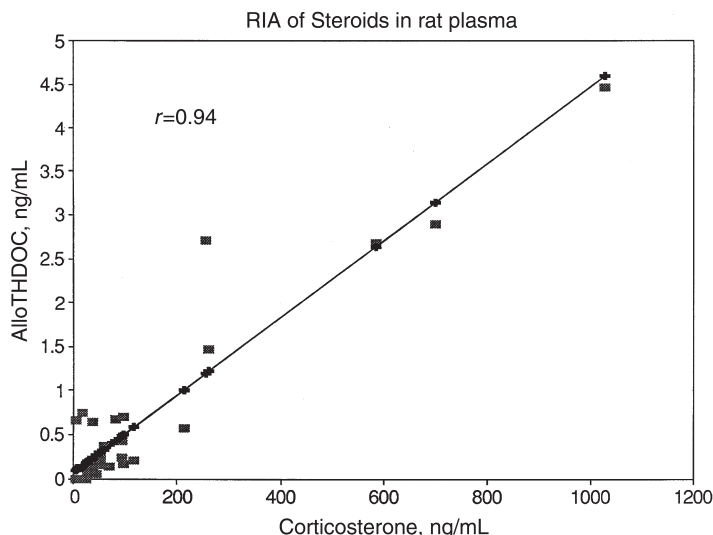


Figure 4.2 The relationship between plasma levels of alloTHDOC and corticosterone in adult male Sprague–Dawley rats before and at various times during 30 min of immobilization stress. The rectangles are the values measured by RIA. The crosses are the values for the calculated regression line for $r = 0.94$. (Data from R. H. Purdy and N. Hagino, unpublished.)

present if the levels of alloTHDOC in brain are the result of synthesis from deoxycorticosterone (DOC) or allopregnanolone or both.

The above 3α -hydroxysteroids enhanced GABA-stimulated chloride flux in rat brain synaptoneurosomes at nanomolar concentrations [38] and interacted with known modulatory sites on GABA_A receptors in a noncompetitive manner [39, 40]. These pregnane neurosteroids have a saturated steroid A ring (see Fig. 4.3 for the steroid four-ring structures of the four epimeric pregnanolones formed in primates).

Notably, the interaction of pregnane neurosteroids with GABA_A receptors was stereospecific, in that the two key features necessary for activity were a 5α - or 5β -reduced steroid A ring and a 3α -OH group. The 3β analogues (i.e., 3β -hydroxy, 5α - or 5β -reduced pregnanes; epiallopregnanolone and epipregnanolone, respectively; Fig. 4.3) were devoid of activity or exhibited a partial inverse agonist profile. Progesterone, estradiol, corticosterone, 5α -dihydrotestosterone, and cholesterol were inactive *in vitro*. In the case of alloTHDOC, the 5β isomer (i.e., 5β -THDOC) had a partial agonist pharmacological profile [41]. Metabolism of $3\alpha,5\alpha$ -THP to allopregnanediol also yielded a steroid with efficacy as a partial agonist [42]. Among all the endogenously occurring steroids examined, $3\alpha,5\alpha$ -THP was the most potent, followed by its 5β stereoisomer (pregnanolone or $3\alpha,5\beta$ -THP) and alloTHDOC [40]. Importantly, the positive modulatory effect of neurosteroids at GABA_A receptors was relatively specific, in that these steroids did not interact with any other known neurotransmitter receptor in the nanomolar-to-low-micromolar concentration range.

Electrophysiological studies revealed that GABAergic steroids facilitated the open state of the GABA-gated channel (frequency and duration, nanomolar concentrations) and could directly activate GABA_A receptors in the absence of GABA at higher (micromolar) concentrations [43]. For a more detailed description of the

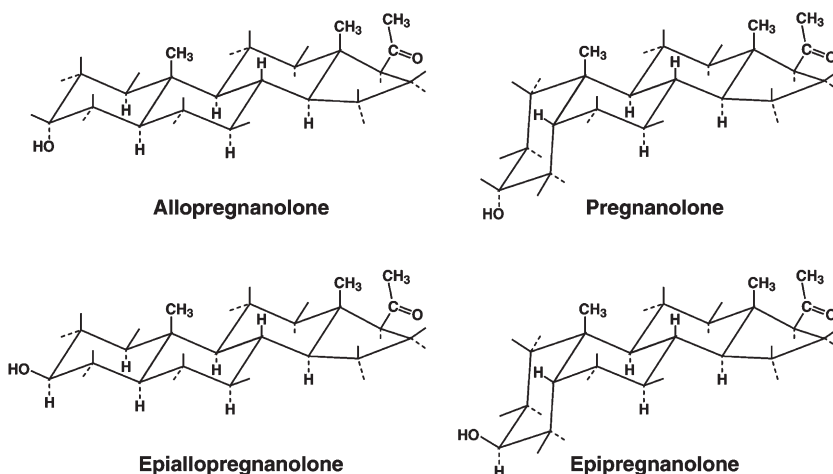


Figure 4.3 Structures of four epimeric pregnanolones. In the two allo (5α) steroids, the 3-hydroxyl group is axial in allopregnanolone (3α) and equatorial in epiallopregnanolone (3β), whereas in the 5β steroids the reverse is the case, with the 3-hydroxyl group being equatorial in pregnanolone (3α) and axial (3β) in epipregnanolone.

perturbation of GABA-gated ion channel kinetics by neurosteroids, the reader is referred to several excellent reports [44–46]. In general, the stereospecificity and enantioselectivity required for neuroactive steroid modulation of GABA_A receptors are features that would be consistent with a specific steroid binding site on GABA_A receptors [46–49]. However, use of recombinant subunit expression studies in conjunction with site-directed mutagenesis has not defined a steroid binding pocket on GABA_A receptors. Thus, the molecular mechanism(s) underlying the perturbation of GABA_A receptor function by neuroactive steroids remains to be determined.

Steroids with GABA-negative actions also have been reported (e.g., PREGS and DHEAS). Whereas PREGS and DHEAS antagonized GABA-gated chloride uptake and conductance in a noncompetitive manner [50, 51], PREGS also markedly reduced glycine-activated chloride currents [52] and enhanced *N*-methyl-D-aspartate (NMDA)-gated currents and elevations in intracellular calcium (Ca^{2+}) [53, 54]. Recent work also demonstrated that the $\text{C}_{3,5}$ reduction of cortisol, which is a primary regulator of the stress response in humans, generated compounds with negative modulatory activity at GABA_A receptors [55]. Notably, $3\alpha,5\beta$ -cortisol inhibited muscimol-stimulated chloride uptake with an median inhibitory concentration (IC_{50}) of $13\ \mu\text{M}$, whereas a $10\ \mu\text{M}$ concentration of cortisol, 11-deoxycortisol, 5α -dihydrocortisol, $3\alpha,5\alpha$ -cortisol, and $3\alpha,5\beta$ -11-deoxycortisol exhibited weak negative modulatory activity on muscimol-stimulated chloride uptake. Therefore, cortisol metabolism may produce steroids with GABA-negative action.

A possible role for pregnane 3-sulfate steroids in the modulation of NMDA receptor function also has been described. Although pregnanolone ($3\alpha,5\beta$ -THP; $50\ \mu\text{M}$) failed to alter NMDA induced cation influx through the NMDA receptor, its 3-position sulfate derivative dose dependently inhibited this NMDA receptor current in chick spinal cord neurons with an EC_{50} of $62\ \mu\text{M}$ [56], and it suppressed NMDA-mediated increases in intracellular Ca^{2+} in rat hippocampal neurons with an IC_{50} of

37 μM [57]. Similarly, 100 μM concentrations of either 3 α ,5 α -THP-sulfate or epipregnanolone-sulfate antagonized NMDA receptor cation conductance by 25 and 50%, respectively [58]. Pregnan-3-sulfates were found to modulate NMDA receptor function in a non-competitive fashion [56, 57], and their inhibitory interaction with this receptor likely involved a specific and separate binding site from positive modulators such as PREGS and related steroids [58–60]. Notably, sulfated and nonsulfated pregnane neurosteroids also had opposing effects on GABA_A receptor function [61]. Thus, even though sulfation of steroids is a major enzymatic reaction of metabolism, secretion, and homeostasis of steroids in the periphery, it may have a role greater than merely facilitating secretion because it also can change the pharmacological activity of steroids in the CNS [29]. While the role of sulfation in the modulation of excitatory and inhibitory ion channels by neurosteroids has yet to be demonstrated *in vivo*, it is possible that the addition and removal of the sulfate group could be a critical control point for neurosteroid modulation of neurotransmitter receptors [62].

In addition to the potent modulation of GABA_A receptor function by pregnane steroids, evidence also indicates that these steroids can interact with other ionotropic receptor systems at micromolar concentrations. Progesterone, 5 α -DHP, and 3 α ,5 α -THP inhibited $^{86}\text{Rb}^+$ efflux through neuronal nicotinic acetylcholine (nACh) receptors derived from mouse thalamus with apparent K_i values of 38, 5.3, and 17.5 μM , respectively [63]. In most cases, neurosteroid antagonism of nACh receptors *in vitro* was observed at concentrations between 10 and 100 μM —levels that are three- to five-fold greater than those estimated to be present in mammalian brain [64]. The inhibition of nACh receptors by progesterone occurred in a noncompetitive manner, did not require the presence of cognate ligand, and was likely dependent on the receptor subunit combinations expressed [65–67]. The attachment of the side chain with a β orientation at the 17 position of the neurosteroid imparted greater inhibitory potency at nACh receptors, whereas, in contrast to GABA_A receptors, the orientation of the hydroxyl group at the 3 position had little impact upon neurosteroid efficacy [68]. Although the sensitivity of various nACh receptor subunit combinations to neurosteroids has yet to be examined, it appears unlikely that a physiologically relevant concentration of pregnane steroids could alter nACh receptor activity.

Progesterone dose dependently inhibited serotonin-evoked currents through serotonin type 3 (5-HT₃) receptors from rat nodose ganglion with an EC₅₀ of 31 μM , whereas its A-ring-reduced metabolite 3 α ,5 α -THP (50 μM) exhibited no observable effect on this measure [69]. In contrast, *in vitro* studies suggest that pregnane steroids are functional antagonists at 5-HT₃ receptors. 3 α ,5 α -THP (10 μM) attenuated serotonin-mediated current by 30–35% in HEK 293 cells [70], while alloTHDOC and alphaxalone inhibited 5-HT-induced [^{14}C]-guanidinium influx in N1E-115 cells with IC₅₀ values of 19 and 44 μM , respectively [71]. Similar to neurosteroid interactions at nACh receptors, the substituent at the 3 position of the steroid A ring had little consequence for the antagonist profile of pregnane neurosteroids at 5-HT₃ receptors [70]. The degree of stereospecificity in neurosteroid modulation of 5-HT₃ receptors remains ambiguous, especially when considering a recent finding that the potency of neurosteroids in inhibiting 5-HT₃ receptor-mediated current was correlated with their lipophilicity [71], thereby suggesting a mechanism involving disruption of lipids adjacent to the receptor.

T-type (low-voltage-activated) Ca^{2+} channels, which act over a range of membrane potentials near the resting potential of most cells and are thought to play an important role in controlling cellular excitability [72–75], can be blocked by micromolar concentrations of $3\alpha,5\alpha$ -THP and alphaxalone [76]. The authors also demonstrated that the 5α -reduced neuroactive steroids were potent peripheral analgesic agents [76]. Notably, structural modifications that eliminated the blocking effect on T-type Ca^{2+} channels but maintained GABA_A receptor potentiation resulted in a complete loss of peripheral analgesic effects. Taken in conjunction with data indicating that GABA_A receptors play a role in centrally mediated effects of neuroactive steroids [77], it is likely that the peripheral analgesic action of 5α -reduced neuroactive steroids is mediated primarily by T channels and only to a smaller extent by GABA_A receptors.

Recent reports documenting neurosteroid interactions with metabotropic sigma 1 (σ_1) receptors are noteworthy. Importantly, the pregnane neurosteroids $3\alpha,5\alpha$ -THP and epipregnanolone were devoid of σ_1 receptor activity [78]. In contrast, progesterone has been identified as one of the most potent neurosteroid inhibitors of agonist binding to the σ_1 receptor [78, 79]. Furthermore, blockade of progesterone's conversion to its pregnane metabolites via a 5α -reductase inhibitor (finasteride) resulted in attenuated agonist binding to the σ_1 receptor, presumably due to augmented levels of progesterone [80]. Clearly, pregnane neurosteroids play little if any physiological role in modulating σ_1 receptor function.

Therefore, $3\alpha,5\alpha$ -THP, alloTHDOC, and $3\alpha,5\beta$ -THP, the three most potent pregnane neurosteroids characterized to date, have nanomolar potencies at GABA_A receptors, and consequently, these actions undoubtedly have physiological significance [18]. Interactions of pregnane neurosteroids at nACh, 5-HT₃, and NMDA receptors occur within the range 10–100 μM and are unlikely to have physiological relevance, even under challenge conditions (i.e., stress or pregnancy).

4.2.3 Enantiomeric Selectivity of Neuroactive Steroids

Studies of the binding and electrophysiological activity of neuroactive steroids have demonstrated that the effect of GABAergic steroids does not result from steroid binding at the benzodiazepine, barbiturate, picrotoxin, or GABA sites of these receptor complexes. These results have led to the concept of unique binding sites for a group of neuroactive steroids on GABA_A receptor complexes [10, 61, 81, 82]. Although there is no current demonstration of the effective binding of a radioactive steroid ligand to such sites, a significant correlation has been obtained between physiological and behavioral effects and *in vitro* structure-activity relationships of GABAergic steroids [83].

Specificity in the interaction of neuroactive steroids with neurotransmitter systems is most convincingly demonstrated by the results of Covey et al. [47] with non-naturally occurring enantiomers of both positive and negative modulators of GABAergic neurotransmission. Allopregnanolone ($3\alpha,5\alpha$ -THP) and pregnanolone ($3\alpha,5\beta$ -THP) occur naturally in one enantiomerically pure form, as illustrated in Fig 4.3. The mirror images of these compounds, known as ent steroids, are shown in Fig 4.4. and were prepared by total synthesis. Here the optically active centers at carbon atoms 3, 5, 8, 9, 10, 13, 14, and 17 are opposite to the configurations in the natural steroids (originally isolated from human pregnancy urine). High entioselectivity was

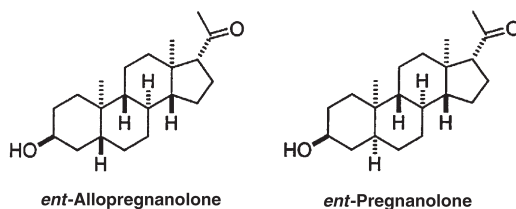


Figure 4.4 Enantiomeric structures of *ent*-allopregnanolone and *ent*-pregnanolone. These synthetic compounds are both much less potent positive modulators of GABA_A receptors when compared to their natural enantiomers shown in Fig. 4.3. [47].

found for $3\alpha,5\alpha$ -THP when compared to *ent*- $3\alpha,5\alpha$ -THP. However, the entioselectivity for $3\alpha,5\beta$ -THP was markedly lower than that for $3\alpha,5\alpha$ -THP, consistent with different binding sites for 3α -hydroxy- 5α - and 3α -hydroxy- 5β -pregnanes. This difference was confirmed by the use of the first specific inhibitor of $3\alpha,5\alpha$ -THP, 3α -hydroxy- 5α -androst-16-ene-17-phenyl, which only weakly affects the *in vitro* and *in vivo* activity of $3\alpha,5\beta$ -THP [84].

4.2.4 Behavioral Effects

Neurosteroids that are positive modulators of GABA_A receptors possess anesthetic [85], hypnotic [86], anticonvulsant [17, 87–93], and anxiolytic [93–100] properties [13]. While $3\alpha,5\beta$ -THP was equipotent with $3\alpha,5\alpha$ -THP as an anxiolytic [95, 98, 101], it was much less potent as an anticonvulsant [101]. The 3β -stereoisomer of $3\alpha,5\alpha$ -THP was inactive in all tests. The effects of pregnane neurosteroids on cognitive function also have been extensively evaluated on conditioned learning and spatial learning tasks [102]. Regardless of the paradigm utilized (e.g., win-shift foraging paradigm, Morris water maze, Y-maze discrimination task), $3\alpha,5\alpha$ -THP, $3\alpha,5\beta$ -THP, and epipregnanolone exhibited a behavioral profile associated with working (spatial) memory deficits [102–105]. Collectively, these behavioral responses closely follow the anticipated patterns based on *in vitro* evidence and suggest that GABAergic steroids modify the functioning of central GABA_A receptors *in vivo*. Therefore, if the findings with exogenous administration of $3\alpha,5\alpha$ -THP are indicative of GABA_A receptor sensitivity to endogenous $3\alpha,5\alpha$ -THP concentration, then endogenous neurosteroids may participate in the physiological control of central nervous system (CNS) excitability.

4.3 EFFECTS OF NEUROSTEROIDS IN ANIMAL MODELS OF ANXIETY AND STRESS

Rodent models of human generalized anxiety symptoms take advantage of a variety of behaviors within the natural behavioral repertoire of rodents that have been interpreted to be “anxiety-like.” While these tasks are based on putative parallels between human and rodent symptoms of anxiety, it is not known whether a rodent experiences “anxiety” in the same way as humans. With this in mind, different behavioral principles seem to underlie various animal models of anxiety such that paradigms can be based on spontaneous exploration, a learned response, conflict

behavior, or a combination of these ideas. Most of these tasks utilize approach–avoidance behaviors in rodents that can provide a good indication of a rodent’s response to conflict in the natural environment. A number of detailed reviews are available on this topic [106–111]. Here, we will briefly describe tasks that have been well validated in terms of their specificity for anxiety-like behaviors and the ability to demonstrate anxiolytic effects with a variety of GABAergic neuroactive steroids. The paradigms described below are good choices for testing hypotheses related to the modulatory effect of neuroactive steroids on anxiety-related behavior. However, these tests also can be influenced by factors unrelated to anxiety. It is therefore recommended that any animal model be tested on more than one anxiety test, so that different sensory and motor modalities can be examined. Alternatively, anti-anxiety effects in multiple tasks that measure both increases and decreases in responding can control for motor effects.

4.3.1 Elevated-Plus Maze

One of the most well-validated animal models of anxiety is the elevated-plus maze task [112–115], as it is able to detect both anxiolytic and anxiogenic agents in rodents [113]. This task is based on a rodent’s natural avoidance of open, elevated alleys that was described almost 50 years ago [116]. Thus, the test sets up a conflict for the rodent between its tendency to explore a novel environment and its tendency to avoid the aversive properties of an open, elevated environment. Typically, mice and rats perform similarly on the elevated-plus maze, although performance on this task can be influenced by light level [117].

The apparatus consists of two open and two enclosed horizontal perpendicular arms of the same size and shape that are at right angles to each other. These four arms extend from a central platform (approximately 5×5 cm), forming the shape of a plus, and are elevated approximately 50 cm above the floor. The two open arms are simple runways with a minimal lip that allows the animal to see the edge without falling off the arm. The enclosed arms are closed runways with high walls that can be opaque. Typically, an animal is placed on the central platform (i.e., at the junction of the four arms) and allowed to explore freely for 5 min. During the 5-min test period, the number of entries into the open and closed arms as well as the amount of time spent in the open and closed arms is measured. Ideally, for an arm entry to be measured, all four paws should be within the arm. If the task is videotaped, additional anxiety-related behaviors can be quantified (see below) [107, 118]. However, the most commonly reported index of anxiety with this task is the percentage of open-arm entries or open-arm time, which is a proportion of the total number of entries or total time of the test, multiplied by 100. Normally, mice prefer the closed arms of the elevated-plus maze. Anxiolytic drugs typically increase the proportion of open-arm entries and the time spent on the open arm [93, 112, 118–122], whereas anxiogenic drugs or treatments decrease the percentage of open-arm entries or time [113, 123].

Comprehensive profiling of behavior also can be achieved by performing detailed videotaped analysis of untreated rodents on the elevated-plus maze, yielding a catalog of readily identifiable behaviors that have been referred to as *risk assessment* [107]. Examples include rearing, head dipping, stretched attend postures, closed-arm returns (i.e., doubling back in, rather than leaving, a closed arm), and several non

exploratory behaviors [118]. “Protected” versus “unprotected” forms of these behaviors can be differentiated based on wall contact. This distinction is based on recent evidence that the most important feature producing open-arm avoidance is the absence of thigmotactic cues (i.e., walls), rather than the height of the maze [123]. Thus, this ethological approach provides multidimensionality to the analysis of elevated-plus maze behavior [107].

An important consideration for interpretation of elevated-plus maze data is that any treatment that affects motor behavior may influence the percentage of open-arm entries or time spent in the open arms. This is due to the fact that the paradigm requires the animal to locomote for a period of 5 min. However, the total number of entries as well as number of entries into the closed arms will give an indication of whether the treatment is influencing activity per se, and closed-arm entries are the most validated measure of activity [106, 124]. In this case, independent paradigms that specifically analyze locomotor behavior are recommended.

Consistent with a GABA-agonist pharmacological profile, the neurosteroids $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP exhibit significant anxiolytic effects on the elevated plus-maze following systemic (1–20 mg/kg, i.p.) [93, 98, 100, 125] or intracerebroventricular (1.25–10 μ g, i.c.v.) [95] administration. Both steroids significantly increased the proportion of open-arm entries and open-arm time. A similar anxiolytic effect was observed following administration of alloTHDOC (5–20 mg/kg, i.p.) [100]. Detailed behavioral analyses demonstrated that the anxiolytic effects of alloTHDOC, $3\alpha,5\alpha$ -THP, and $3\alpha,5\beta$ -THP did not reliably decrease measures of risk assessment and were not associated with a change in activity level, in contrast to the anxiolytic profile of a 1-mg/kg dose of diazepam [100]. However, in one study, a decrease in total entries, taken as an index of sedation, was observed following administration of a 10 μ g (i.c.v.) dose of $3\alpha,5\alpha$ -THP [95]. Systemic administration of allopregnanediol, a partial agonist at GABA_A receptors, produced anxiolytic effects within a dose range of 10–40 mg/kg, i.p. [126]. Both systemic and i.c.v. administration of the 3β -stereoisomer of $3\alpha,5\alpha$ -THP ($3\beta,5\alpha$ -THP) had no effect on elevated-plus maze behavior or locomotor activity [95, 100], consistent with reports of its inability to potentiate GABA-gated currents. Therefore, GABA agonist neurosteroids produce anxiolytic effects in the rodent elevated-plus maze, and their anxiolytic profile can be partially distinguished from that of a well-characterized benzodiazepine (i.e., diazepam).

Recent findings in progesterone receptor (PR) knockout mice indicate that the anxiolytic effect of progesterone was due to its conversion to $3\alpha,5\alpha$ -THP [127]. With this mouse model, a targeted null mutation of the PR gene eliminated the function of both PR-A and PR-B subtypes of the PR. Administration of progesterone (50 and 75 mg/kg) or $3\alpha,5\alpha$ -THP (2 and 5 mg/kg) to PR knockout and wild-type mice significantly increased open-arm entries and time. Notably, the anxiolytic effect of progesterone was blocked by pretreatment with finasteride (50 mg/kg), a 5α -reductase inhibitor that prevents the conversion of progesterone to $3\alpha,5\alpha$ -THP, in both genotypes [127]. Finasteride pretreatment did not alter $3\alpha,5\alpha$ -THP's anxiolytic effect in either genotype. These results indicate that the anxiolytic action of progesterone does not require PRs, but does require its conversion to $3\alpha,5\alpha$ -THP.

In contrast, neurosteroids that are noncompetitive antagonists of GABA_A receptors have mixed effects on anxiety-related behavior. For example, systemic administration of DHEA and DHEAS exhibited anxiolytic effects on the elevated-plus maze [128], whereas pregnenolone had anxiogenic properties [129]. PREGS had

a biphasic effect on elevated-plus maze behavior, exhibiting anxiolytic effects at low doses and anxiogenic effects at increasing doses [129]. Although data for DHEAS and PREGS are limited, it is possible that the mixed effects of these GABA antagonist steroids reflect confounding effects on activity level or the reduced ability of sulfated steroids to cross the blood–brain barrier.

Recent findings indicate that microinjection of GABA agonist neuroactive steroids or agents that increase neurosteroid levels into the hippocampus can produce anxiolytic effects. Specifically, microinjection of 2.5 and 5- μ g doses of pregnanolone (3 α ,5 β -THP) into the dorsal hippocampus of male rats significantly increased the proportion of time spent in the open arms of the elevated-plus maze [130]. Similar behavioral effects were seen when the hippocampus was microinjected with agonists of peripheral mitochondrial benzodiazepine receptors, which led to an increase of brain 3 α ,5 α -THP [131].

With regard to the neurobiology of stress and anxiety, the role of the hippocampus has been described as a “comparator system” that can detect whether a threat is familiar or novel, thus requiring either a conditioned automatic response or higher order processing, respectively [132]. The septum, posterior cingulate, and thalamic nuclei also have been implicated in this role [133]. Overall, the hippocampus is important in traumatic memory consolidation and, with the entorhinal cortex, in contextual fear–conditioned behaviors. Projections from the hippocampus to the bed nucleus of the stria terminalis (BNST) and from the BNST to hypothalamic and brain stem sites may be involved in the expression of contextual fear conditioning (see Section 4.3.4).

4.3.2 Geller–Seifter and Vogel Conflict Tests

Both of these paradigms are conflict tests that utilize operant responding for a food reward [134] or a water reward [135], with electric shock as the aversive component in both tasks. The number of lever presses for either the food or water reward, in conjunction with a mild shock that is delivered on a fixed-ratio schedule, is the variable of interest. Anxiolytic drugs typically increase the number of lever presses, or the number of shocks that are acceptable, for the food or water reward. Although weeks of training on the lever-press task are required, these paradigms have the advantage that once stable performance baselines are established, the same animal can be used repeatedly to examine drug effects by comparing these data with its baseline (i.e., it is its own control). These tasks also have the advantage of an additional internal control, unpunished responding, that gives an indication of nonspecific drug effects. While both the Geller–Seifter and Vogel paradigms have been used primarily in rats, there are data on neurosteroid effects in mice as well.

Early work in Sprague–Dawley rats and Swiss Webster mice demonstrated that both alloTHDOC (10, 15, and 20 mg/kg, i.p.) and 3 α ,5 α -THP (20 mg/kg, i.p.) had anxiolytic effects in the Vogel and Geller–Seifter conflict tests, respectively [94, 97]. Both steroids significantly increased punished responding without significantly influencing unpunished responding. However, a trend for sedation was found following the highest dose of alloTHDOC. Subsequent studies have confirmed these initial findings and demonstrated that systemic administration of 3 α ,5 α -THP [98, 99], 3 α ,5 β -THP [98], and alphaxalone [96] significantly enhanced punished drinking, consistent with their positive modulatory effects at GABA_A receptors.

Another study compared the anxiolytic potential of four pregnanediols, differing only in the stereochemical orientation (α or β) of the steroid A ring and the 20-OH group [126]. The effects of these pregnanediols were examined in the Vogel test following i.c.v. administration and compared to those of their 20-ketone analogs (i.e., $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP). All four pregnanediols significantly enhanced punished drinking at doses ranging from 10 to 60 μg , whereas $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP enhanced punished responding when administered at 2.5- and 5- μg doses, respectively [126]. Doses of $3\beta,5\alpha$ -THP up to 100 μg were inactive in the Vogel test, consistent with this steroid's lack of activity at GABA_A receptors. These results suggest that some endogenously occurring pregnanediol metabolites (e.g., Fig. 4.3) also may influence physiological processes related to anxiety via an action at GABA_A receptors, in addition to the actions of their parent steroids $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP.

As discussed in chapters on the neurobiology of stress and anxiety disorders, the amygdala plays a pivotal role in the assessment of and response to danger. The amygdala has extensive connections to the cortex and locus ceruleus and projections to the striatum, hypothalamus, midbrain, and brain stem. Thus, it can exert control over locomotor, neuroendocrine, autonomic, and respiratory responses. In addition, the amygdala also is seen as the common pathway and processor of "fear" [136]. Recent work demonstrated that infusion of $3\alpha,5\alpha$ -THP (8 μg per side) into the central nucleus of the amygdala (CeA) of male and ovariectomized (OVX) female rats produced a significant anxiolytic effect, measured by a significant increase in punished responding in the modified Geller Seifter conflict test [137]. Likewise, bilateral infusion of $3\alpha,5\alpha$ -THP (8 μg per side) into the CeA significantly increased open-arm time and open-arm entries on the elevated plus maze, compared to vehicle-infused controls. Bilateral infusion of the noncompetitive GABA_A receptor steroid antagonists PREGS (0.0018 μg side) or DHEAS (2–8 μg per side) into the CeA had no effect on punished or unpunished responding, nor did i.c.v. administration of these two steroids at doses ranging from 1 to 20 μg [137]. Collectively, these findings suggest that the CeA is a key region involved in the mechanisms underlying the anxiolytic effects of $3\alpha,5\alpha$ -THP.

4.3.3 Light–Dark Box

Conceptually similar to the elevated-plus maze, the light–dark exploration task is based on the conflict between a rodent's tendency to explore a novel environment and the aversive properties of a brightly lit open field [138, 139]. The apparatus is an acrylic box divided by a panel into a large open area (28 cm long \times 28 cm wide) and a slightly smaller enclosed area that is painted black (28 cm long \times 19 cm wide). The open area is illuminated to 355 lux, whereas the light level in the dark chamber is low (2 lux). Typically, the animal is placed in the center of the light area, facing away from the entrance to the dark chamber, and behavior is recorded for 5–10 min. The number of transitions between the light and dark chambers, the time spent in both chambers, as well as the latency to enter the dark chamber, can be quantified by photocell array or manually. A chamber crossing is defined as all four paws of the mouse inside the chamber. The number of transitions and the time spent in the light chamber are the most commonly used variables. Anxiolytic drugs increase the number of transitions as well as the amount of time spent in the light chamber, relative to the dark chamber, which are believed to be indicators of the animal's willingness to explore the brightly lit open area [94, 139].

Interpretation of the light–dark box data can be confounded by effects of treatments on locomotor activity, particularly when the number of transitions is the only variable that is utilized. This appears to be more of a problem with anxiogenic drugs or treatments, as low levels of general activity have been shown to produce false positives [140]. Thus, it is recommended that independent testing for locomotor function be conducted in new classes of drugs in order to detect stimulant or sedative properties that could confound interpretation of the light–dark transition measure.

Early work found that alloTHDOC (7.5–15 mg/kg, i.p.) significantly increased the number of light–dark transitions in Swiss Webster mice [94]. There was no decrease in generalized locomotion, although a trend for sedation was found following administration of a 30-mg/kg dose of alloTHDOC. Subsequent studies in Swiss Webster mice documented dose-dependent anxiolytic effects of alloTHDOC (20 and 40 mg/kg, i.p.) and $3\alpha,5\alpha$ -THP (10–40 mg/kg, i.p.) with the light–dark box and that the 3β stereoisomer of $3\alpha,5\alpha$ -THP was inactive in this task [97, 98].

4.3.4 Acoustic Startle and Fear-Potentiated Startle

It was over 50 years ago that conditioned fear could be revealed by the magnitude of the startle response to an auditory stimulus [141]. This fear-potentiated startle paradigm involves pairing a neutral stimulus (e.g., light) with a footshock prior to the auditory stimulus [136, 142]. During testing, startle is elicited by the auditory stimulus alone (normal acoustic startle) or the auditory stimulus in the presence of the footshock-paired neutral stimulus (potentiated startle). The fear-potentiated startle effect only occurs following prior light–shock pairings, and not when lights and shocks have been presented in an unpaired or random fashion [143]. Presenting the conditioned stimulus repeatedly without further light/shock pairings will extinguish the prior fear conditioning (i.e., enhanced startle response) [144]. Notably, anxiolytic drugs reduce fear potentiated startle, whereas drugs that induce anxiety in people will increase potentiated startle in rodents [136].

Intracerebroventricular administration of corticotropin-releasing factor (CRF) can increase the amplitude of the acoustic startle response [145–147], whereas CRF peptide antagonists exhibit anxiolytic effects in the fear-potentiated startle paradigm [137, 147, 148]. Recently, it was reported that CRF-potentiated startle was significantly reduced in OVX female rats treated acutely or chronically with progesterone as well as in lactating female rats (high progesterone levels) [149]. Administration of $3\alpha,5\alpha$ -THP (10 mg/kg, i.p.) had a similar effect, suggesting that the effect of progesterone to reduce CRF-potentiated startle might be due to its metabolism to $3\alpha,5\alpha$ -THP. However, neither chronic progesterone nor $3\alpha,5\alpha$ -THP attenuated fear-potentiated startle [149]. Taken in conjunction with work indicating that the BNST, but not the CeA, is required for the elevation of startle after CRF infusion [150], the disparity in the effects of progesterone and $3\alpha,5\alpha$ -THP on CRF- versus fear-potentiated startle may reflect differential sensitivity of these brain regions to the steroids.

4.3.5 Mirrored Chamber

The chamber-of-mirrors procedure was first described in 1990 [151] as a measure of anxiety in rodents. This test generates an approach–avoidance conflict behavior which is based on early work [152] indicating that several vertebrate species demonstrate

behavioral responses to mirrors such as aggressive threats and approach–avoidance behavior. The chamber consists of a mirrored cube 30 cm on a side which is open on one side and placed inside the center of an opaque Plexiglas box (40 × 40 × 30.5 cm). Thus, a 5-cm corridor completely surrounds the five-sided mirrored chamber. A sixth mirror is placed against the container wall opposite the single open side of the mirrored chamber. With the exception of the initial report in which mice were tested for 30 min [151], mice are placed in the corridor outside the mirrored chamber. Latency to enter the mirrored chamber, number of entries, time per entry, and total time in the mirrored chamber are recorded for a period of 5–10 min [153–156]. In this assay, a mouse avoids entering the mirrored chamber, whereas it will enter readily if the walls of the chamber are not mirrored [151]. However, a recent study in three inbred strains of mice in which the mirrors in the chamber were replaced with white or gray tiles suggested that a brightness or position effect in the chamber also could explain the avoidance behavior in certain genotypes [156]. Nonetheless, the mirrored-chamber procedure has been validated in terms of being responsive to a wide spectrum of anxiolytic agents at doses that do not affect locomotor activity [151, 153–155]. Recently, a modified version of the mirrored chamber was validated in the mouse to detect both anxiolytic and anxiogenic agents [157].

Neurosteroids also exhibited anxiolytic effects, measured by the behavior of mice in the mirrored test of anxiety [155]. Administration of progesterone (1–10 mg/kg) or its reduced metabolite 3 α ,5 α -THP (0.5 and 1 mg/kg) produced a dose-dependent anxiolytic response, measured by increased number of entries and total time spent in the mirrored chamber as well as by decreased latency to enter the chamber. Consistent with the GABA agonist profile of 3 α ,5 α -THP, the anxiolytic effect was blocked by coadministration of picrotoxin, a GABA_A receptor chloride channel antagonist. In contrast, administration of a neurosteroid with GABA antagonist properties (DHEAS, 1 and 2 mg/kg) produced an anxiogenic response, measured by a decrease in the number of entries and total time spent in the mirrored chamber as well as an increase in the latency to enter the chamber. Therefore, both anxiolytic and anxiogenic effects of neurosteroids have been demonstrated that are consistent with their activity at GABA_A receptors.

4.3.6 Open-Field Activity

Open-field activity is the oldest and simplest measure of rodent emotional behavior [158, 159]. Briefly, spontaneous exploratory locomotion, proximity to the walls and central arena, and number of fecal boli deposited are quantified in a brightly lit novel open field for a period of 5–10 min. Whereas fully automated systems are widely used, the scoring also can be performed manually. Thigmotaxis is measured by comparing activity in the center with activity in the perimeter of the open field. An animal exhibiting high perimeter and low center activity would be interpreted as possessing high levels of anxiety. However, an important distinction is that open-field activity is not a specific measure of anxiety or as specific a measure of anxiolytic drug response, compared to other tasks described in this section. Nonetheless, open-field activity does provide a useful measure of normal versus abnormal exploratory behavior. With this *proviso* in mind, early work documented that systemic administration of 3 α ,5 α -THP and alloTHDOC (20 mg/kg, i.p.)

significantly increased activity in the open-field test, whereas the 3 β epimer of 3 α , 5 α -THP was inactive [97].

4.3.7 Defensive Burying Behavior

Defensive burying refers to rodent behavior of displacing bedding material to remove or avoid aversive (unfamiliar and/or harmful) objects from their habitat. Together with flight, freezing, and fighting, defensive burying is part of the behavioral repertoire of unconditioned defensive reactions in an animal [160]. The shock-prod defensive burying procedure was first described in 1978 by Pinel and Treit [161]. Using a familiar test chamber, the animal is confronted with a wire-wrapped prod (or probe) that is inserted through a small hole in one of the test chamber walls so that the prod is approximately 2 cm above the bedding. Following the first contact with the electrified prod (in which the animal receives an electric shock), the animal's behavior is either observed or videotaped for a test session of 10–15 min. Typically, rodents will cover the shock source with cage litter or bedding. The latency and time to the first prod contact (shock) and the latency time to initiate burying behavior are recorded. The most commonly used measures of this defensive behavior have been the frequency and duration of time spent on prod-directed burying behavior as well as the height of the bedding material around the shock prod. In general, anxiolytic drugs suppress conditioned defensive burying, as measured by a reduction in the mean duration of burying behavior [160].

As with other animal models of anxiety-related behaviors, an ethological analysis of several concurrent and competitive behavioral indices of fear/anxiety, avoidance, reactivity, and exploration can provide a better understanding of the full defensive behavioral repertoire in rodents [160]. The following behavioral categories can be distinguished: ambulation, rearing, immobility, burying, grooming, prod-explore (i.e., oriented toward prod in a stretch/attend-like posture), and eating/drinking. One benefit of this ethological analysis stems from the fact that there is considerable variation across individual animals and studies in the mean time that animals engage in burying, which also represents only a small portion (between 3 and 30%) of the behaviors exhibited during the 10–15-min test. Thus, in addition to measuring defensive burying, one can quantify other avoidance/defensive behaviors (i.e., decreased prod exploration, increased freezing/immobility) as well as general exploratory and other behaviors (i.e., ambulation, rearing, grooming, and consummatory activities). While this paradigm has the advantage of requiring much less ambulatory locomotor activity than other tasks, it does have the potential confound that treatments producing behavioral sedation may produce false positives on this task (i.e., decreased defensive burying due to sedation). This task also has been well characterized for rats but not for mice.

The GABAergic neuroactive steroids 3 α ,5 α -THP (0.25–1.0 mg/kg) and 3 α ,5 β -THP suppressed burying behavior in this task, consistent with their anxiolytic profile [125, 162]. Progesterone (1–4 mg/kg) also was effective, whereas PREGS (1–4 mg/kg) was not [162]. Additional studies determined that microinjection of 3 α ,5 β -THP into the dorsal hippocampus or the lateral septum significantly decreased burying behavior [130]. These microinjection studies are consistent with data indicating that the posterior parts of the septal region, the dorsal hippocampus, the caudal shell of the nucleus

accumbens, and the dorsal raphe nuclei are critical parts of the neural circuitry underlying defensive burying [160].

4.3.8 Separation-Induced Ultrasonic Vocalizations

The rat pup ultrasonic vocalization procedure has been shown to detect a wide range of anxiolytic and anxiogenic compounds [163]. In rat pups, vocalizations in the frequency range of 35–45 kHz are produced in the first two postnatal weeks and are associated with social isolation. Most investigators count the number of calls using a microphone sensitive to high-frequency sounds connected to a signal detection device, such as a bat detector, that transduces the sounds into the audible range. However, it also is recommended that nonvocal variables such as changes in ambient and body temperature, locomotion, and coordination should be monitored to determine whether the effects of a treatment are specific for vocal behavior [163]. Variations of this procedure have been referred to as maternal separation, early deprivation, or social isolation [164].

Initial studies determined that benzodiazepine agonists (such as diazepam and chlordiazepoxide) decreased the number of ultrasonic vocalizations in isolated rat pups at doses lower than those that produced muscle relaxation and sedation [165]. The GABAergic neurosteroids $3\alpha,5\alpha$ -THP and alloTHDOC also significantly decreased the number of ultrasonic vocalizations in isolated rat pups [166–169]. Repeated administration of alloTHDOC to infant rats during postnatal days 2–10 prior to recurring separation from their mothers significantly attenuated the increase in anxiety-related behavior that was evident in rats administered vehicle, when the animals were tested on the elevated-plus maze as adults [168]. In the study by Patchev et al. [168], repeated maternal separation also produced neuroendocrine alterations related to dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis in adult rats, and these endocrine responses were significantly reduced in the animals administered repeated alloTHDOC as infants. In contrast, a study that utilized a slightly different maternal separation procedure did not observe a difference in anxiety related behavior in adult rats that had been repeatedly separated from their mothers as infants versus non-isolated controls [169]. However, as adults, non-isolated controls treated daily with $3\alpha,5\alpha$ -THP during postnatal days 2–6 exhibited less anxiety-like behavior than all other groups, including isolated rats treated with $3\alpha,5\alpha$ -THP. Postnatal treatment with $3\alpha,5\alpha$ -THP also eliminated the sex difference in anxiety-related behavior that was apparent in non-injected adult rats. In general, these studies suggest that chronic treatment with the GABAergic neurosteroids alloTHDOC and $3\alpha,5\alpha$ -THP may offset the behavioral and neuroendocrine changes that occur as a result of adverse early-life events.

4.3.9 Modified Forced-Swim Test

The forced-swim test is considered an animal model of depression, rather than of anxiety. Since many of the core symptoms of depression are difficult to measure in laboratory animals, many of the available animal models of depression are based on the actions of known antidepressants or on the responses to stress [170]. The forced-swim test was initially developed in the rat [171] and later in the mouse [172]. In this model, rodents are forced to swim in a confined environment. The animals initially

swim around and attempt to escape and eventually assume an immobile position. On subsequent tests, the latency to immobility is decreased [170, 173]. The immobility is thought to reflect either a failure of persistence in escape-directed behavior (i.e., behavioral despair) or the development of passive behavior that disengages the animal from active forms of coping with stressful stimuli. A drug or treatment that produces an increase in immobility would be considered to produce depressive-like behavior, whereas a drug that increases escape-directed behavior (i.e., decreased immobility) would be considered to have an antidepressant effect. However, one drawback of the Porsolt forced-swim test is that it is not sensitive to the selective serotonin reuptake inhibitor (SSRI) class of antidepressant compounds.

In an effort to enhance the sensitivity of the forced-swim test in rodents so that it could be SSRI responsive, several simple procedural modifications were made [173]. Increasing the water depth to 30 cm produced less immobility time because animals could not contact the cylinder bottom with their paws. Rating the predominant behavior over 5-s intervals allowed the distinction of the following behaviors: climbing, horizontal swimming, and immobility. With regard to the putative antidepressant effects of neurosteroids, systemic (0.5, 1 or 2 mg/kg, i.p.) or i.c.v. (1 or 2 μ g per mouse) administration of $3\alpha,5\alpha$ -THP dose dependently reduced the duration of immobility in the modified Porsolt forced-swim test [174, 175]. This antidepressant-like effect of $3\alpha,5\alpha$ -THP was potentiated by pretreatment with the GABA_A receptor agonist muscimol [174] or ethanol [175], whereas it was blocked by pretreatment with the GABA_A receptor antagonist bicuculline. Administration of progesterone (2 mg/kg) to OVX female rats also was effective at producing effects in the forced-swim test that were similar to that of the tricyclic antidepressants [176]. The ability of progesterone to decrease immobility by increasing climbing behavior was comparable to that observed during days 14 and 17 of pregnancy. Thus, physiological states in which progesterone levels are high can produce antidepressant-like effects, presumably due to progesterone's reduction to $3\alpha,5\alpha$ -THP.

Chronic ethanol withdrawal, which has been reported to decrease endogenous $3\alpha,5\alpha$ -THP levels (see Section 4.4.4), produced an increase in immobility on the modified forced-swim test [175]. Notably, subthreshold doses of $3\alpha,5\alpha$ -THP (0.25 or 0.5 μ g per mouse) reversed the depressive-like symptoms associated with ethanol withdrawal. Recent findings also indicated that withdrawal from daily progesterone injections significantly increased immobility in the modified forced-swim test in female DBA/2 mice (E. H. Beckley and D. A. Finn, unpublished results). There was a further increase in immobility when the metabolism of progesterone was blocked with administration of finasteride, suggesting that withdrawal from progesterone or progesterone metabolites produced an increase in depressive-like behavior. These findings are consistent with recent work suggesting that some symptoms of progesterone withdrawal are due to withdrawal from $3\alpha,5\alpha$ -THP [177, 178]. Although speculative at this point, these findings are consistent with an inverse relationship between fluctuations in endogenous $3\alpha,5\alpha$ -THP levels and depressive-like symptoms (i.e., \uparrow $3\alpha,5\alpha$ -THP, \downarrow depression; \downarrow $3\alpha,5\alpha$ -THP, \uparrow depression).

4.3.10 Mild Mental Stress Models and Social Isolation

As described in Section 4.1, acute exposure of male rodents to a variety of stressors rapidly increased brain $3\alpha,5\alpha$ -THP and alloTHDOC to the equivalent of 10–30 nM

concentrations [14, 15]. Recent work also demonstrated that immobilizing rats on their backs for 20 min produced a significant increase in brain $3\alpha,5\alpha$ -THP levels as well as in levels of the precursor steroids pregnenolone, progesterone, and 5α -DHP [179]. Notably, when the vocalizations of the rats were recorded during the period of immobilization and subsequently played for 1 h to a group of unrestrained rats (referred to as mild mental stress or “din stress” by the authors), there was a slight but significant increase in brain $3\alpha,5\alpha$ -THP, 5α -DHP, progesterone, and pregnenolone levels in 66% of the rats [179]. Thus, acute exposure to physical stressors or stressors encompassing a psychological component can increase the synthesis of GABAergic neuroactive steroids to levels that should facilitate GABAergic neurotransmission in the brain.

In contrast, long-term social isolation after weaning significantly decreased brain $3\alpha,5\alpha$ -THP and alloTHDOC levels in male rodents [180–182]. There is conflicting evidence on the specific point(s) in the neurosteroid biosynthetic pathway where social isolation might be altering GABAergic neurosteroid levels. Whereas one study demonstrated that social isolation also decreased brain pregnenolone and progesterone levels [181], a second study provided evidence that the effect of social isolation occurred at a point downstream from progesterone [182]. In the study by Dong et al. [182], social isolation did not alter progesterone levels, but it significantly decreased the expression and protein levels of the enzyme 5α -reductase (see Fig. 4.1 for biosynthetic pathway). Nonetheless, the decreases in GABAergic neurosteroid levels were associated with an increase in anxiety-like behavior, measured by a significant decrease in percentage of open-arm time on the elevated-plus maze and a significant decrease in punished responding in the Vogel conflict test [181]. Social isolation also produced a decrease in biochemical and electrophysiological measures of GABA_A receptor function [181] as well as decreased sensitivity to the hypnotic effect of GABAergic compounds [180, 183]. Thus, the period of exposure to stress may produce opposite effects on endogenous neuroactive steroid levels in the brain.

4.4 NEUROACTIVE STEROID INTERACTIONS WITH STRESS-INDUCED BEHAVIORS

From the results summarized in Section 4.3, it is apparent that GABA agonist neurosteroids have anxiolytic properties. However, their role as endogenous modulators of anxiety is less clear. For example, systemic administration of progesterone has anxiolytic effects, measured by elevated-plus maze behavior, via its metabolism to $3\alpha,5\alpha$ -THP, since inhibiting the enzymatic conversion of progesterone to $3\alpha,5\alpha$ -THP blocked the anxiolytic effect of progesterone [184]. Likewise, results from a recent study suggest that the ability of progesterone to attenuate CRF-enhanced acoustic startle response might be due to progesterone's metabolism to $3\alpha,5\alpha$ -THP, since administration of $3\alpha,5\alpha$ -THP had a similar effect, whereas use of a progestin that was not metabolized to $3\alpha,5\alpha$ -THP (i.e., medroxy-progesterone) did not [149]. While the putative modulatory role of brain versus peripheral-derived $3\alpha,5\alpha$ -THP on anxiety-related behavior is not clear, recent work has examined the effect of ligands that stimulate biosynthesis of $3\alpha,5\alpha$ -THP in adrenalectomized–gonadectomized (ADX–GDX) and intact animals [131, 185]. These ligands, which activate peripheral benzodiazepine receptors, significantly increased brain levels of $3\alpha,5\alpha$ -THP to a

greater extent in the intact versus ADX–GDX rats, yet the increase in brain 3 α ,5 α THP also was associated with a marked anti-conflict effect in the Vogel test. Thus, it is possible that peripheral sources of 3 α ,5 α THP may have a greater impact in modulating anxiety-related behavior than brain-derived 3 α ,5 α THP.

4.4.1 HPA Axis and Stress

Acute stress of many types results in the release of CRF, adrenocorticotrophic hormone (ACTH), and cortisol in humans and corticosterone in rodents (see Fig. 4.5) [186]. Corticosteroids bind to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), which are colocalized in brain regions important in the regulation of anxiety, such as the hippocampus, septum, and amygdala. A recent report indicated that increased corticosterone levels in rodents were highly correlated with measures of risk assessment [187]. Several animal models of anxiety have demonstrated that both MR and GR can modulate specific aspects of anxiety, as antagonists for both receptor subtypes have been shown to have anxiolytic effects [186].

GRs are found in higher concentrations in regions involved in feedback regulation of the hormonal stress response. GRs have a 10-fold lower affinity for corticosterone than MRs, becoming occupied only during stress and at the circadian peak, whereas

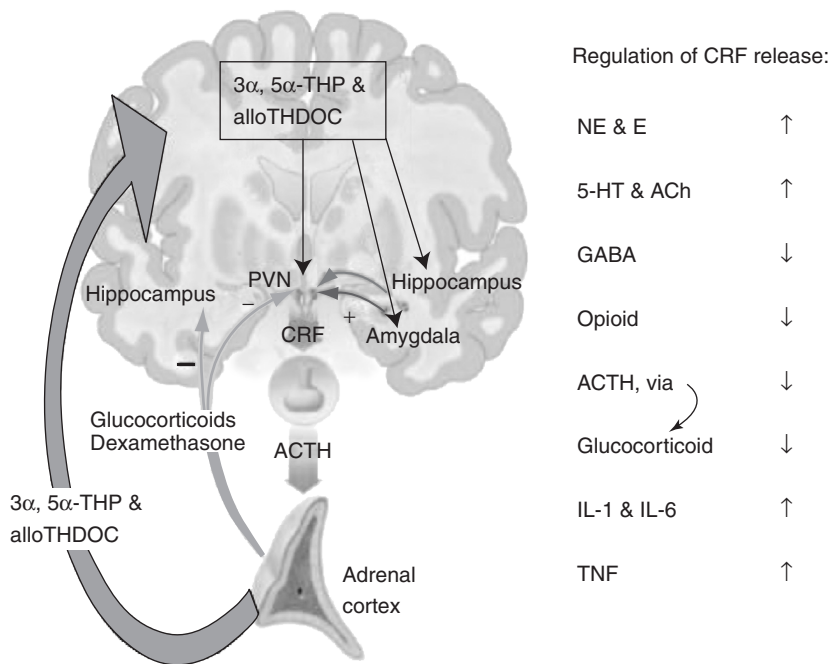


Figure 4.5 Various points of control for regulation of the HPA axis by neurosteroids, neurotransmitters, ACTH, glucocorticoids, and the immune system[186, 222, 224, 284–286]. A variety of stressors activate the HPA axis, which is typically observed as an increase in glucocorticoids from the adrenal cortex. Stress also produces an increase in levels of the GABAergic neurosteroids 3 α ,5 α -THP and alloTHDOC in the brain via the circulation and *de novo* synthesis. (Adapted from Nestler et al. [170].) (See color insert.)

MR is almost completely occupied under basal conditions. Since it is believed that GR action is mainly regulated by hormone level, with MR action being influenced by receptor density, endogenous corticosteroids can play an important role in the maintenance of homeostatic equilibrium via its two receptors [188, 189]. High corticosteroid levels, such as those elicited by exposure to an acute stressor, decrease the number of GRs, which in turn results in increased corticosterone secretion and feedback resistance.

One of the many neurotransmitters that can modulate HPA axis activity is the GABAergic system, as the GABAergic innervation of CRF-producing cells of the hypothalamic paraventricular nucleus (PVN) has been shown to be particularly important for the control of HPA axis activity [190]. For example, GABAergic function in the PVN was increased after ADX and was normalized when corticosteroid levels were restored [191]. A series of elegant studies recently determined that corticosterone can modulate GABAergic function in PVN neurons within hours in a mode different from that of neurosteroids (i.e., presynaptic vs. postsynaptic effect) [192]. Restraint stress was found to decrease GABAergic inhibition by 50%, suggesting that the corticosterone-induced changes in GABAergic function in the PVN also occur with natural fluctuations in this hormone level due to stress exposure.

Adding another level of complexity to regulation of the HPA axis, acute stressors and activation of the HPA axis also can increase levels of the GABAergic neurosteroids $3\alpha,5\alpha$ -THP and alloTHDOC (see Figs. 4.2 and 4.5 and Section 4.1). One possibility is that a stress-induced increase in GABAergic neurosteroids could participate in the adaptation to the stress response. Consistent with this idea, administration of $3\alpha,5\alpha$ THP reduced anxiety that was induced by CRF [193] in addition to exerting actions within the hypothalamus to dampen the activity of the HPA axis [193, 194].

4.4.2 Anxiety-Related Disorders

Alterations in HPA axis function are suggested in posttraumatic stress disorder (PTSD), as shown by a decrease in cortisol levels and increased suppression of cortisol levels with the GR agonist dexamethasone (dexamethasone suppression test, DST), suggesting enhanced sensitivity to cortisol feedback [195, 196]. Patients with PTSD also had a blunted ACTH response to CRF versus control subjects, suggesting hypersecretion of CRF [197]. Consistent with this observation, patients with combat-related PTSD had elevated levels of CRF in the cerebrospinal fluid (CSF) [198]. However, more recent work suggested that women with PTSD do not differ from traumatized subjects or non-traumatized controls in basal cortisol levels [199]. When the subjects were challenged with a dose of ACTH that had been reported to elicit cortisol responses equivalent to that seen following extreme naturalistic stressors, the cortisol responses were significantly increased in the patients with PTSD versus other subjects. An independent study determined that, following a CRF challenge dose, cortisol levels determined hourly in CSF over a 6 h period were significantly elevated in patients with PTSD compared to matched control subjects [200]. Whereas baseline DHEA levels did not differ between the patients in the study by Rasmusson et al. [199], the increase in DHEA in response to the ACTH challenge was significantly greater in the PTSD patients. The authors hypothesized that the anti-glucocorticoid properties of DHEA might antagonize glucocorticoid negative feedback within the

brain and pituitary, which would ultimately result in an increase in the adrenal capacity for cortisol release due to a facilitation of CRF or ACTH release.

CSF levels of CRF have also been shown to be elevated in patients with obsessive compulsive disorder [201], but not panic disorder [202]. However, an additional study demonstrating no difference in baseline CSF levels of CRF between control subjects and patients with generalized anxiety disorder, panic disorder, or obsessive-compulsive disorder [203] suggests that these patients did not exhibit tonic hypersecretion of CRF. Therefore, evidence for dysfunction of CRF or HPA systems in anxiety disorders has been inconsistent [204].

A recent study by Strohle and colleagues in patients with panic disorder [205] demonstrated that panic attacks induced by sodium lactate and cholecystokinin tetrapeptide were accompanied by marked decreases in circulating levels of 3α , 5α -THP and 3α , 5β -THP, with concomitant increases in the neuroactive steroid antagonist 3β , 5α -THP. Similar changes were not observed in control subjects. Baseline 3α , 5α -THP levels in these patients were increased [206], particularly in the early follicular phase of the menstrual cycle [207]. It is possible that during an induced panic attack patients with panic disorder fail to maintain compensatory increased 3α , 5α -THP levels. Therefore, neuroactive steroids may play a role in the pathophysiology of panic disorders.

Early work suggested that examination stress produced a significant increase in the density of peripheral benzodiazepine receptors (PBRs), compared to unstressed controls [208]. Although PBRs play a role in the translocation of cholesterol from the outer to the inner mitochondrial membrane [209], which represents the rate-limiting step for the synthesis of neuroactive steroids (see Fig. 4.1) [210], the physiological consequence of the increased PBR with regard to neuroactive steroid biosynthesis was not known. More recent work determined that PhD examination stress increased heart rate, blood pressure, plasma 3α , 5α -THP, and cortisol levels and the density of PBR when compared with baseline levels [211]. The increase in plasma cortisol was not correlated with the increase in PBR density, suggesting that the acute stress-induced increase in cortisol levels did not depend on rapid biosynthesis. Notably, the increase in plasma 3α , 5α -THP was significantly positively correlated with increased PBR density. Although no causal relationship can be drawn from these findings, it is possible that use of synthetic PBR agonists to enhance the synthesis of 3α , 5α -THP could be useful in the treatment of anxiety disorders [211].

A number of studies in which various animal models of anxiety were utilized demonstrated that injection of CRF into the locus ceruleus, paraventricular nucleus of the hypothalamus, BNST, and CeA produced signs and symptoms that were identical to animals in response to stress as well as those observed in patients with anxiety disorders [137, 148, 212–215]. Notably, central CRF systems, in addition to those that participate in the control of the HPA axis, contribute to affective behavior regulation [216]. In male rats, alfaxalone, the 11-keto derivative of 3α , 5α -THP, was shown to attenuate the anxiogenic behavioral effects of CRF and swim stress without reducing the CRF-induced increases in plasma ACTH [217]. Alfaxalone also inhibited the startle enhancing effects of CRF in male rats without disrupting CRF-stimulated locomotor activity [218]. At doses of 5 and 10 μg i.c.v., 3α , 5α -THP counteracted the anxiogenic action of 5 μg CRF in male rats and prevented the adrenalectomy-induced upregulation of CRF gene expression [193]. Both

progesterone and $3\alpha,5\alpha$ -THP attenuated CRF-enhanced acoustic startle, whereas the progestin medroxyprogesterone acetate enhanced CRF-induced startle [149]. Thus, CRF appears to play a significant role in anxiety-related and stress-related states that can be attenuated by GABAergic neuroactive steroids. Collectively, the data also suggest that agents acting at CRF receptors may have therapeutic value in anxiety disorders. Indeed, the development of CRF receptor antagonists as novel anxiolytics is being actively pursued (see chapter 5) [215].

For a more detailed discussion of neuroactive steroids in psychopathology and menstrual cycle-linked CNS disorders, the reader is referred to the reviews by Dubrovsky [219], and Backstrom et al., [220] and chapter 21 in volume I of this handbook.

4.4.3 Depression

Evidence suggests that stressful life events can produce hyperactivity of the HPA axis and can increase vulnerability to depression [170, 221–225]. In general, patients with major depressive disorder exhibit elevated plasma cortisol and CRF levels, with approximately 50% of patients displaying an impaired suppression of cortisol secretion after dexamethasone administration (DST test) [225]. Patients with stress-associated disorders such as depression appear to escape from glucocorticoid negative feedback, resulting in a persistent activation of the HPA axis.

Protracted social isolation in rodents produces behavioral symptoms also found in depression, in addition to producing a significant decrease in cortical $3\alpha,5\alpha$ -THP levels and a decrease in expression of the biosynthetic enzyme 5α -reductase [182]. Taken in conjunction with the finding that $3\alpha,5\alpha$ -THP has antidepressant properties [174], manipulation of neuroactive steroid biosynthesis may have therapeutic benefits. Studies in patients with unipolar depression found a 60% decrease in $3\alpha,5\alpha$ -THP content in CSF or plasma, compared to values in normal subjects [226–228]. Treatment with fluoxetine, a broad-spectrum antidepressant that inhibits serotonin reuptake, for 8–10 weeks normalized the levels of $3\alpha,5\alpha$ -THP in the depressed patients. Improvement in symptomatology and the increase in $3\alpha,5\alpha$ -THP concentrations in CSF were significantly, positively correlated in the study by Uzunova et al. [227]. It was subsequently determined that fluoxetine altered $3\alpha,5\alpha$ -THP biosynthesis by increasing the affinity of 5α -DHP for the enzyme 3α -hydroxysteroid dehydrogenase (see Fig. 4.1) [229]. Data in rats indicate that the effect of fluoxetine on $3\alpha,5\alpha$ -THP biosynthesis was rapid; significant increases in brain $3\alpha,5\alpha$ -THP levels were observed between 15 and 120 min post injection of fluoxetine [230]. This ability of fluoxetine to shift the activity of the 3α -hydroxysteroid dehydrogenase enzyme toward the reductive direction (i.e., toward the formation of $3\alpha,5\alpha$ -THP from 5α -DHP) provides evidence for a possible role of neuroactive steroids in successful antidepressant therapy.

There are massive levels of progesterone ($\sim 5 \times 10^{-7}$ M) in maternal plasma during human pregnancy near term. These levels are much greater (5- to 50-fold) than those that occur in most other pregnant mammals, including non-human primates [231]. It is therefore not surprising that the combined levels of $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP approach 15 ng/mL or $\sim 5 \times 10^{-8}$ M near term (Fig. 4.6), although there are wide individual variations. There is only a very limited transfer of progesterone from maternal plasma into the fetus, but it does enter the

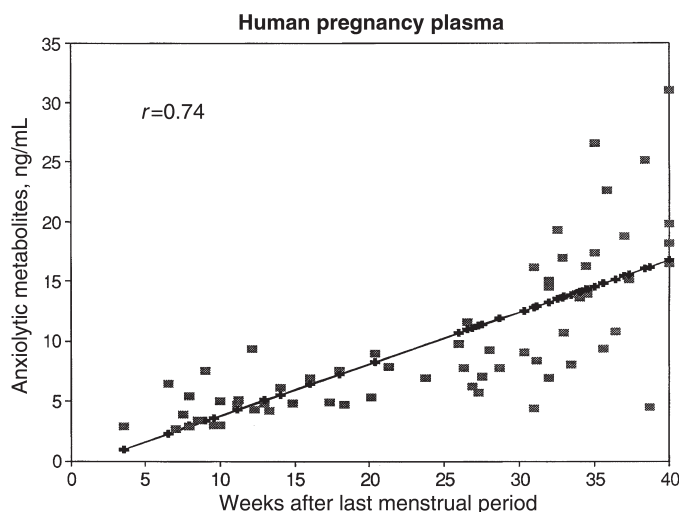


Figure 4.6 Combined levels of anxiolytic metabolites of progesterone, allopregnanolone, and pregnanolone, measured in 11 normal women during pregnancy. The weeks of gestation were established from last menstrual period. The levels of the anxiolytic metabolites were measured in duplicate by specific RIA after extraction of serum and separation of the individual neuroactive steroids as described by Purdy et al. [21] and Schmidt et al. [287]. (Data from R. Purdy and D. Castracane, unpublished.)

fetal circulation from the placental syncytiotrophoblast where it is synthesized and then converted in the fetus to $3\alpha,5\alpha$ -THP and its sulfate [232].

The etiology of depression in childbearing women is poorly understood, but it has been suggested that the very large changes in progesterone and its anxiolytic metabolites in the postpartum period may predispose some women to depression [233, 234]. These dramatic postpartum changes are illustrated in Fig. 4.7 as the dramatic logarithmic decrease in concentrations of progesterone, $3\alpha,5\alpha$ -THP, and $3\alpha,5\beta$ -THP in the maternal plasma of three normal women. A more detailed time profile of pregnanolone isomers in the postpartum period has been provided by Hill et al. [235]. Gilbert Evans et al. [236] have found a significant reduction in the concentrations of $3\alpha,5\alpha$ -THP plus $3\alpha,5\beta$ -THP in pregnant women with a history of postpartum depression at 36–38 weeks, regardless of their current state of depression. Their results suggest that depression history was associated with an underlying dysregulation of these neuroactive steroids.

4.4.4 Acute and Chronic Effects of Alcohol

Acute and chronic administration of alcohol also activates the HPA axis, measured by an increase in plasma corticosterone in rodents [237–241]. Elegant work by the Rivier laboratory demonstrated that i.p. or intragastric administration of alcohol produced a rapid, significant increase in plasma ACTH levels [242] and neuronal activity in the cell bodies of the PVN of the hypothalamus that express CRF and vasopressin [238, 239]. Since available *in vivo* evidence had not consistently

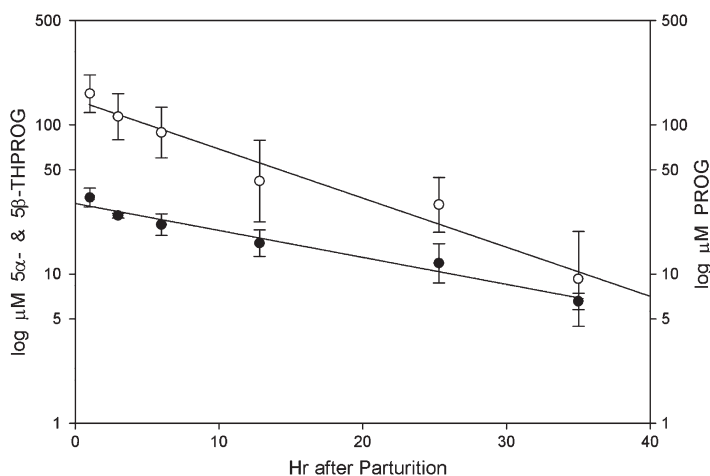


Figure 4.7 Logarithm of sum of micromolar concentrations (\pm standard error of the mean) of progesterone (PROG, open circles) and sum of its two anxiolytic metabolites (closed circles), allopregnanolone (5α -THPROG) and pregnanolone (5β -THPROG), obtained in the plasma from three women up to 36 h after parturition. The anxiolytic metabolites were measured by RIA as described in Fig. 4.6. Progesterone was measured by RIA in duplicate using a standard kit from ICN (Costa Mesa, CA). (Data from R.H. Purdy and D. Castracane, unpublished.)

demonstrated whether acute administration of alcohol influenced pituitary activity, independent of CRF and vasopressin, the mechanism by which ethanol stimulated the HPA axis was uncertain. However, recent findings shed some light on the specific sites of action of ethanol on the activity of the HPA axis. Specifically, i.c.v. and i.p. administration of alcohol increased the expression of proopiomelanocortin (POMC) in the anterior pituitary with a time course that corresponded to ACTH release [243]; these responses were completely abolished by immunoneutralization of CRF and vasopressin. This finding provides strong evidence that the stimulatory effect of acute alcohol administration on the HPA axis requires the release of endogenous CRF and vasopressin, rather than a direct pituitary influence of alcohol on the corticotropes.

Acute administration of anesthetics, including ethanol, also has been shown to increase plasma levels of progesterone, the parent steroid of $3\alpha,5\alpha$ -THP, in male rats [244]. This initial finding was recently extended with the demonstration that acute injection of ethanol doses ranging from 1 to 4 g/kg significantly increased cortical $3\alpha,5\alpha$ -THP levels to pharmacologically active concentrations in rats and mice [245–247]. Acute alcohol intoxication also increased plasma $3\alpha,5\alpha$ -THP levels in male and female adolescent humans [248, 249]. Due to the fact that the pharmacological profile of ethanol is similar to that of $3\alpha,5\alpha$ -THP, it has been suggested that certain behavioral effects of ethanol and $3\alpha,5\alpha$ -THP might share a GABAergic mechanism [250, 251]. Taken in conjunction with the demonstration that manipulating endogenous $3\alpha,5\alpha$ -THP levels altered specific behavioral and physiological effects of ethanol (i.e., \uparrow $3\alpha,5\alpha$ -THP, \uparrow ethanol effect; \downarrow $3\alpha,5\alpha$ -THP, \downarrow ethanol effect) [246, 252], these data suggest that an ethanol-induced increase in endogenous $3\alpha,5\alpha$ -THP levels might potentiate or prolong certain behavioral effects of ethanol via its

action at GABA_A receptors. Since additional studies demonstrated that the endocrine response to acutely administered alcohol was principally due to steroid biosynthesis in adrenal and gonadal tissue [253–255], it is possible that endogenous 3 α ,5 α -THP levels might play a role in the modulation of the excitability of the HPA axis in response to stress (see Fig. 4.5) and in the modulation of cortical 3 α ,5 α -THP concentration after ethanol injection.

Electrophysiological recordings from CA1 pyramidal neurons determined that the action of ethanol on GABA_A receptor-mediated inhibitory postsynaptic current amplitude was biphasic [256]. Initially, there was a rapid, direct effect of ethanol on GABA_A receptor activity, followed by an indirect effect that was believed to be mediated by the synthesis of 3 α ,5 α -THP. Furthermore, it has been clearly demonstrated by these electrophysiological methods that ethanol increases GABAergic transmission at both presynaptic and postsynaptic sites in rat central amygdala neurons [257], as well as altering glutamatergic transmission in this tissue [258]. This poses the possibility of neuroactive steroid action at both of these sites of neurotransmission within the amygdala.

4.4.5 Stress-Induced Drug Reinstatement

Drug relapse is a major problem in the treatment of drug addiction in humans [259]. Early work in monkeys and rodents used a reinstatement procedure as an animal model of drug relapse [260–263]. In the reinstatement model, laboratory animals initially are trained to lever press for access to drugs in operant chambers, which produces robust self-administration behavior. The drug-reinforced responding is extinguished when saline is substituted for the drug solution or when the infusion pump is disconnected (i.e., the animal stops pressing the lever and responding to the stimuli previously associated with drug delivery). During reinstatement tests, animals are given access to the levers, but the drug remains unavailable, making it possible to test for the ability of various events to reinstate drug-seeking behavior. The results from a number of laboratories have determined that the following events are extremely effective in promoting operant-reinforced responding on the lever previously associated with drug: reexposure to the drug (i.e., priming injection), acute exposure to stressful stimuli (e.g., footshock), and environmental stimuli (e.g., cues) previously associated with drug self-administration [264–268].

In the 1990s, many studies demonstrated that exposure to intermittent footshock was at least as effective as priming injections of cocaine, heroin, or nicotine and even more effective than priming injections of alcohol in promoting high levels of responding in tests of reinstatement over a range of footshock durations (10–60 min) [269–273]. Administration of CRF (0.3 and 1 μ g, i.c.v.) potently reinstated heroin-seeking behavior, whereas the CRF antagonist α -helical CRF_{9–41} (3 and 10 μ g, i.c.v.) attenuated stress-induced but not heroin-induced reinstatement [274]. Additional studies determined that ADX and corticosterone replacement did not alter footshock-induced reinstatement to alcohol [275], whereas the nonselective CRF antagonist D-Phe-CRF_{12–41} (0.3 and 1 μ g, i.c.v.) and the nonpeptide CRF₁ receptor antagonist CP-154,526 (15–30 mg/kg, subcutaneous) significantly decreased reinstatement of alcohol-, heroin-, and cocaine-seeking behavior that was induced by intermittent footshock [275, 276]. These data suggest that extra hypothalamic CRF systems are involved in stress-induced drug reinstatement behavior.

With regard to neurosteroid effects on drug reinstatement, a single study has demonstrated that priming injections of $3\alpha,5\alpha$ -THP (3 and 7.5 mg/kg, i.p.) dose dependently reinstated previously extinguished responding for ethanol but not for sucrose [277]. In contrast, conditioned stimuli reinstated previously extinguished ethanol- and sucrose-seeking behavior, suggesting that the mechanisms that subserve cue-induced reinstatement do not depend on the nature of the positive reinforcer tested. However, the fact that $3\alpha,5\alpha$ -THP selectively promoted responding for ethanol after a period of abstinence suggests that GABA_A receptor modulation may contribute to the processes involved in reinstatement of ethanol-seeking behavior.

Notably, several neuroactive steroids also modulate ethanol self-administration behavior. The initial study determined that $3\alpha,5\alpha$ -THP significantly increased ethanol-reinforced operant responding in male rats [278] by enhancing responses during the initial run of a 30-min operant session. A subsequent study determined that $3\alpha,5\alpha$ -THP selectively enhanced ethanol-reinforced operant responding when a sucrose solution was concurrently available [279], suggesting specificity for $3\alpha,5\alpha$ -THP in modulating ethanol self-administration in male rats. Consistent with these findings, $3\alpha,5\alpha$ -THP dose dependently increased ethanol preference drinking during the first hour of 2-h limited-access sessions in male mice [280, 280a]. In contrast to the results with $3\alpha,5\alpha$ -THP, epipregnanolone and a novel neuroactive steroid with a 3α -carboxyl group ($3\alpha,5\beta$ -20-oxo-pregnane-3-carboxylic acid, termed PCA) dose dependently reduced operant ethanol self-administration in male rats [281]. The opposite effect of epipregnanolone and PCA on ethanol self-administration may be related to different inhibitory actions of these compounds on either GABA_A or NMDA receptors, respectively [282, 283]. Thus, dual modulation of inhibitory and excitatory neurotransmitter systems by certain neuroactive steroids may provide a novel therapeutic potential for modifying the reinforcing effects of addictive drugs.

4.5 CONCLUSIONS AND OUTLOOK

There has been an exponential increase in basic science and clinical investigations of neuroactive steroids in the last two decades. Methodological advances in the specificity, sensitivity, and accuracy of measurement of nonconjugated neuroactive steroids are still continuing. The chemical nature of the “sulfate-like” esters of PREG and DHEA in brain has yet to be elucidated, even though these esters are predominant in mammalian blood. Since anesthetic neuroactive steroids such as alfaxalone were introduced five decades ago, there has been a continuing interest in the development of other synthetic neuroactive steroids for the clinical treatment of neurological disorders, including stress and anxiety. Additionally, neuroactive steroids have been linked to pathogenesis in menstrual cycle-linked disorders of the CNS.

Several thousand synthetic steroids have been assayed for their interactions as allosteric modulators of neurotransmitter systems. New compounds with unusual properties continue to be reported. Yet, in our opinion, universal recognition of the neurophysiological and neuropharmacological importance of neuroactive steroids awaits definitive demonstration of their clinical importance and therapeutic advantage.

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5

EMERGING ANXIOLYTICS: CORTICOTROPIN-RELEASING FACTOR RECEPTOR ANTAGONISTS

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5.1	Introduction	177
5.2	CRF Receptor/Ligand Family	179
5.2.1	Ligands for CRF Receptor Family	180
5.2.2	Subtypes and Distribution of CRF Receptor	181
5.3	Peptide Ligand Pharmacology of CRF ₁ and CRF ₂ Receptors	185
5.3.1	Mechanisms of Peptide Ligand Binding to CRF ₁ and CRF ₂ Receptors	190
5.4	Nonpeptide Ligands for CRF ₁ Receptors	195
5.4.1	Mechanism of Nonpeptide Ligand Interaction with CRF ₁ Receptor	195
5.5	Potential for Therapeutic Intervention in Anxiety and Depression	196
5.6	Conclusions	198
	References	199

5.1 INTRODUCTION

The corticotropin-releasing factor (CRF) system is widely accepted as the primary mediator of an organism's response to stress. CRF is one of a number of neurohormones synthesized in discrete hypothalamic nuclei where in response to a stressor is secreted into the portal vasculature where it acts directly at the pituitary. At the pituitary, and more specifically at the anterior pituitary, CRF binds to cognate receptors resulting in the release of the proopiomelanocortin-derived peptide adrenocorticotrophic hormone (ACTH). This in turn acts at the adrenal gland to secrete glucocorticoids, and these steroids feed back at the level of the hypothalamus and the anterior pituitary to attenuate the release of CRF and ACTH, respectively. This hormone loop is known as the hypothalamic–pituitary–adrenal (HPA) axis and is tightly regulated in mediating the stress response. In addition to its endocrine effects, CRF is widely distributed, synthesized, and secreted from extrahypothalamic

sites within the central nervous system (CNS) acting on higher centers of the brain where it mediates the appropriate response of the organism to various physical and psychological stressors.

Although the modern concept that hypothalamic secretions were the key mediators of the stress response dates back to the works of Sir Geoffrey Harris and Hans Selye in the early 1950s [1, 2], it was the independent determination from the experiments of Guillemin and Rosenberg [3] and Saffran and Schally [4] that provided the first direct evidence of a hypothalamic factor (CRF) that was responsible, under stressful situations, for regulating the production of ACTH. The identity of this specific factor, however, was not discovered until 30 years later when Vale and colleagues [5] reported the isolation, characterization, synthesis, and *in vitro* and *in vivo* biological activities of CRF from sheep hypothalamus. Once this peptide was characterized, evidence began to accumulate that this stress hormone may play a major role in human affective disorders. Nemeroff and colleagues made one of the first observations implicating this system in depression by demonstrating that the cerebrospinal fluid (CSF) of depressed patients contained elevated levels of the CRF peptide itself, suggesting a hypersecretion of this hormone in the brain during this disease [6]. At the same time, two groups independently demonstrated that in depressed individuals the ACTH response to systemic administration of CRF was blunted, suggesting a downregulation of the system [7–9], and a positive correlation was observed between CSF concentrations of CRF and the degree of post-dexamethasone suppression of plasma cortisol [10]. These data taken together indicated the tight control of a system whereby elevated levels of CRF in the CNS could cause the downregulation or desensitization of the receptors at the pituitary, thereby causing a blunting of the ACTH response upon exogenous administration of CRF. Further corroboration of this hypothesis was presented by the findings that in suicide victims CRF receptor binding sites were significantly decreased in postmortem frontal cortical tissue, again consistent with the mechanism of elevated levels of CRF in brain causing a homologous downregulation of CRF receptors [11].

Examination of CRF in detail presented further evidence that this system plays a key role in the manifestation of anxiety and depression. Levels of CRF in the CNS of depressed individuals who had undergone successful treatment paradigms were found to be altered in a direction consistent with the hypothesis that elevated levels of CRF were manifesting the disease symptomatology. For example, when CRF was measured in the CSF of depressed individuals before and after successful electroshock convulsive therapy (ECT), the elevated levels of CRF in the CSF was found to decrease following ECT and corresponded to patient improvement in mood [12]. Another study, performed on 24 patients suffering from major depression and treated for six to eight weeks with antidepressants, interestingly found that although no differences in the CSF levels of CRF were observed between pre- and posttreatment, despite clinical improvement, those patients who did not relapse in the following six months had a significant reduction in CSF concentrations of CRF while those that relapsed did not. These findings suggest that the hyperactivity of CRF in the CNS may predispose patients to relapse following treatment and are consistent with the findings of a higher risk of relapse in patients that exhibit elevated levels of cortisol following concomitant administration of the CRF/dexamethasone suppression test [13]. Furthermore, while the simple measurement of CRF peptide in the brain may not accurately reflect the hyperactivity of the system in these disorders, clinical

studies to date are in agreement that CRF levels in the CSF generally decrease in parallel with clinical recovery regardless of the treatment paradigm used [14, 15].

More than a decade later, the receptors for this neurohormone were identified and cloned from a variety of species, including human. The ability to look biochemically at the cloned human receptor accelerated the identification and characterization of nonpeptide small molecules that could block these receptors and paved the way for the development of novel therapeutics for disorders associated with a dysregulated stress axis, including anxiety and depression. This chapter provides a brief overview of the CRF system, including the known ligands and receptor subtypes, and details the different interactions between the ligands (both peptide and nonpeptide) and these receptors. With the relatively recent discovery of nonpeptide antagonists for the CRF system, the similarities and differences between the receptor/ligand interactions will be examined and the preliminary evidence and potential for their utility as anxiolytics and antidepressants will be presented.

5.2 CRF RECEPTOR/LIGAND FAMILY

Since the discovery of ovine CRF from the sheep hypothalamus, other functional CRF peptides have been identified from many species, including rat [16], human [17], goat [18], cow [19], pig [20], suckerfish (urotensin-I, isolated from the urophysis extract of the sucker (teleost) fish *Catostomus commersoni* [21, 22]), and xenopus (sauvagine isolated from a skin extract of the South American tree frog *Phyllomedusa sauvegei* [23, 24]). All of these peptides demonstrated the ability to cause the secretion of ACTH from primary cultures of rat anterior pituitary cells [25]. Initially, urotensin-I was thought to subserve the role of CRF in the fish; however, the discovery of two independent CRF genes in this species [26] prompted many laboratories to begin searching for the presence of other CRF-like molecules in mammals. In 1995, with the aid of immunocytochemical methodologies, a urotensin-like molecule was isolated from the Edinger–Westphal nucleus of the rat brain which had high homology (63% sequence identity) to urotensin-I [27]. This new peptide, termed urocortin, was cloned, synthesized, and found to stimulate the secretion of ACTH both in vitro and in vivo. The human homolog of urocortin was subsequently identified by the cloning of its messenger RNA (mRNA) from a human genomic brain library and localized to human chromosome 2 [28]. These initial findings became the basis for the discovery of a family of mammalian urocortin peptides which, as will be described below, have distinct distributions and pharmacological profiles but whose precise relevance to human physiology or pathophysiology is not yet completely understood.

CRF and its related peptides mediate their actions through high-affinity binding sites in various peripheral and central tissues. In addition, a high-affinity soluble binding site exists, the CRF binding protein, which is expressed predominantly in the brain and pituitary and is presumed to modulate the activity of CRF itself [29]. Both the natural peptides and the close analog peptides have been radiolabeled and used in radioligand binding and receptor autoradiographic studies of the distribution of CRF receptors and their role(s) in physiological processes. There have been numerous publications that have described the radioligand binding characteristics of CRF receptors using those available radioligands in a variety of

tissues (for a review see [30–33]). The receptors for CRF exist as two distinct subtypes encoded by separate genes and have been termed CRF₁ and CRF₂. Both receptors belong to the class B family of G-protein-coupled receptors (GPCRs), which includes receptors for secretin, vasoactive intestinal peptide (VIP), calcitonin, and glucagon, among others. CRF₁ receptors positively regulate the accumulation of cyclic adenosine monophosphate (cAMP) in response to CRF in both heterologously expressed systems and native tissues from brain and periphery and are therefore coupled to G_s as the major signal transduction mechanism [34, 35]. The CRF₂ receptor has also been shown to couple through G_s and stimulate the production of cAMP, but thus far only in heterologously expressed cell lines containing the human, rat, or mouse forms [36–38].

5.2.1 Ligands for CRF Receptor Family

Since the discovery of ovine CRF, many peptides from multiple species have been discovered that play similar functional roles as described above. In addition to urocortin 1 (UCN1), two other mammalian peptides have been identified from the mouse and human termed urocortin 2 (UCN2) and urocortin 3 (UCN3) [39, 40]. In the human, these same peptides have been identified as stresscopin (SCP) and stresscopin-related peptide (SRP) [41]. The species homology between mouse and human is 76% for SRP/UCN2 and 90% for SCP/UCN3 and these peptides share approximately 30–40% homology with rat/human (r/h) CRF and urocortin. (for a review see [42]). While UCN1 has equal affinity for both CRF receptor subtypes, UCN2 and UCN3 are selective for the CRF₂ receptor subtype and have little or no affinity for the CRF binding protein (described in detail below).

CRF and the related urocortins have been localized to a variety of tissues within the body. The literature is replete with articles detailing the localization and distribution of CRF and the urocortins both from the mRNA and the protein levels. While a full description is beyond the scope of this chapter, briefly, within the CNS, CRF is widely distributed with the highest levels evident in the hypothalamus, amygdala, cortical regions, and cerebellum (reviewed in [31, 43–47]). In general, the distributions of CRF and the urocortins are remarkably nonoverlapping with one exception and a common localization to hypothalamic structures. UCN1 demonstrates the highest levels of expression in regions such as the Edinger–Westphal nucleus, substantia nigra, lateral superior olive, dorsal raphe nucleus, and hypothalamus [27, 48–50]. UCN2 is also discretely localized to hypothalamic nuclei, specifically the paraventricular, supraoptic, and arcuate nuclei as well as in the brain stem [40]. UCN3 is primarily localized to the hypothalamus and more specifically in the preoptic and paraventricular nuclei. It is also highly expressed in medial amygdala, lateral septum (indicating an association with the CRF₂ receptor), and bed nucleus of the stria terminalis [51]. In addition, these peptides have been localized in a variety of peripheral tissues, including the skin and skeletal muscle [52], human heart and kidney [53], and the colon, suggesting a role for stress-induced gastrointestinal (GI) function [54–56].

To date, there are no known endogenous physiological antagonists for the CRF receptor system, however; various truncations of agonist peptides, with deletions of the first 8–11 N-terminal amino acids, have resulted in potent peptide antagonists, both selective and nonselective with respect to their affinities at the CRF₁ and CRF₂

receptor subtypes. These peptides have shed new light not only on understanding the physiology of the CRF system but also for the elucidation of the discrete interactions of the peptide/ligand complex. As with the agonist peptides either deletions of the C-terminal amino acids or simple deamidation of the carboxy terminus renders these peptides essentially inactive at CRF receptors, suggesting that at some level the binding requirements for these ligands have some similarities between the two receptor subtypes. Deletions of the agonist peptides or modifications in the N-terminus, however, have successfully identified potent and functional receptor antagonists that are both selective and nonselective for the CRF receptor subtypes. Peptides such as α -helical CRF(9–41) [57], d-PheCRF(12–41) [58], and astressin [59] were synthesized and found to be potent functional antagonists at both the CRF₁ and CRF₂ receptor subtypes. Antisauvagine-30 [60], an N terminally truncated form of sauvagine, and the more recent cyclized astressin-2B [61] are two peptides that have approximately 400- to 500-fold selectivity for the CRF₂ receptor over the CRF₁ receptor, and these peptides have been recently used to begin dissecting out specific roles of the CRF₂ receptor subtype in a variety of in vivo studies [62, 63]. As will be discussed below, the peptide interactions with the receptors lie largely with the extracellular N-terminal domain of the receptors, and it is precisely the composition of the N-terminus of the peptides that confers agonist activity. This point is illustrated elegantly by the recent studies examining the interactions of the peptide antagonist astressin and the expressed soluble form of the N-terminus of the CRF₁ receptor [64].

5.2.2 Subtypes and Distribution of CRF Receptor

The current understanding of the subfamily of CRF receptors encompasses two receptor subtypes termed CRF₁ and CRF₂ receptors, with the CRF₂ receptor existing in three identified splice variant isoforms (CRF_{2(a)}, CRF_{2(b)}, and CRF_{2(c)}). Prior to the cloning and elucidation of the subtypes of CRF receptors, the labeled agonists [¹²⁵I]r/hCRF and [¹²⁵I]ovine(o) CRF were used to elucidate the binding characteristics and anatomical distribution of CRF receptors in the brain, and many publications exist describing the binding characteristics of these prototypical labeled compounds (for review see [30–33]). Indeed, early drug discovery efforts utilized these ligands in native brain homogenate preparations (typically rat frontal cortex or cerebellar tissues) to identify nonpeptide molecules as potential antagonists. Fortunately, these early pharmacological characterizations and drug discovery efforts were largely unaffected by the discovery of a second family member (the CRF₂ receptor) by virtue of the fact that the prototypical endogenous peptides [¹²⁵I]r/hCRF and [¹²⁵I]oCRF have lower affinity for the CRF₂ receptor subtype (10–100 nM), making them essentially in vitro selective tools for the CRF₁ receptor under the conditions in which they were being used. The subsequent radiolabeling of the amphibian peptide sauvagine (from frog skin, *P. sauvagei* [23]), which has high affinity for both the CRF₁ and CRF₂ receptor subtypes, allowed for the first time the discrimination of CRF₁ and CRF₂ receptor pharmacology and distribution [65].

The CRF₁ receptor was first cloned from a human corticotropic tumor and characterized as a 415-amino-acid receptor belonging to the class B subfamily of GPCRs with a characteristically large N-terminus, linked to seven putative trans-membrane domains terminating with an intracellular C-terminal tail and reported

along with an alternatively spliced form of which included a 29-amino-acid insert in the first intracellular loop (Fig. 5.1) [66]. This was shortly followed by the cloning of the rat form of this receptor by the same group [67]. Following the cloning and characterization of the CRF₁ receptor the second subtype of this family was identified and termed the CRF₂ receptor [37] and was also subsequently identified from a variety of species [36–38]. This subtype had approximately 71% identity with the CRF₁ receptor and was shown to exist as three independent splice variants differing from each other only at the extreme N-terminal end (see Fig. 5.1). The chromosomal mapping of the human CRF₂ gene has also been determined and has been localized to chromosome 7p21–p15 [68]. As with any rapidly expanding family of proteins, consistency of molecular nomenclature is difficult to maintain. The literature unfortunately contains a number of different designations for this family of receptors, including CRF-RA [67] and PC-CRF [36, 69] for the CRF₁ receptor; CRF_{2 α} [37] for the CRF_{2(a)} receptor; CRF-RB [67], CRF_{2 β} [37], and HM-CRF [36] for the CRF_{2(b)} receptor; and CRF_{2 γ} [70] for the CRF_{2(c)} receptor. In an attempt to maintain some consistency with other GPCR families of receptors, a nomenclature for these subtypes has recently been proposed according to the International Union of Basic and Clinical Pharmacology (IUPHAR) convention for the naming of receptors, and these receptors should be referred to hereafter as CRF₁, CRF_{2(a)}, CRF_{2(b)}, and CRF_{2(c)} receptors [71].

Compared to the CRF₁ receptor, the CRF_{2(a)} receptor is a 411-amino-acid protein with the typical glycosylation sites in the N-terminal domain. The CRF_{2(b)} receptor, which has been cloned from rat, mouse, and human, contains 431 amino acids and differs from the 411-amino-acid CRF_{2(a)} isoform in that the first 34 amino acids in the N-terminal extracellular domain are replaced by a unique sequence 54 amino acids in length [37, 72]. The CRF_{2(c)} receptor has thus far only been identified in the amygdala of the human brain [70]. This splice variant uses yet a different 5' alternative exon for its amino terminus and replaces the first 34-amino-acid sequence of the CRF_{2(a)} receptor with a unique 20-amino-acid sequence (see Fig. 5.1). Very recently a soluble splice variant of the mouse CRF_{2(a)} receptor has been identified and characterized. This soluble protein appears to localize in the same discrete anatomical brain regions of the mouse brain and may represent yet another mechanism through which this system can modulate the activity of its endogenous agonists [73].

There have also been a number of splice variants for the CRF₁ receptor reported in the literature; however, the physiological significance of these variants still remains to be determined. Besides the first 29-amino-acid variant first described in the initial pituitary tumor library described above, a second splice variant for the CRF₁ receptor (CRF_{1 γ}) derived from human hypothalamus has been reported where a large portion of the N-terminal domain is deleted. This protein, however, has lower affinity for agonists and appears to be weakly functional when expressed in mammalian cell lines [74]. Finally, a third splice variant identified from human pregnant myometrium (CRF_{1 δ}) contains a deletion of 14 amino acids in the C-terminus of the seventh transmembrane domain. On heterologous expression of this receptor, it was found that while binding of agonist ligands was largely unaffected, this receptor could not couple through G proteins and was therefore functionally inactive [75]. While these receptor splice variants offer insight into the structure and functional conformations of the CRF₁ receptor, their in vivo

physiological relevance will have to await further mRNA and protein expression determinations in native tissues.

Many studies have described in great detail the anatomical distribution of the CRF receptor subtypes and isoforms in a variety of species using both receptor autoradiographic studies with [125 I]sauvagine or [125 I]oCRF and in situ hybridization studies using probes that are both subtype specific and isoform selective (reviewed in great detail in [76–80]). The highest density of CRF₁ receptors exists in the pituitary gland, where CRF₁ expression clustered on corticotropes related to its effects on the release of ACTH from the anterior lobe of the pituitary [77]. High levels of CRF₁ receptors have been localized in brain to the cerebral cortical areas, amygdala, and hippocampus in rodents and also in hypothalamus and amygdala in nonhuman primates (for review see [77, 80]). There are also a number of peripheral sites to which CRF receptors have been localized, including adrenals, and throughout the immune and reproductive systems where levels of CRF₁ mRNA have been localized in the testis and ovary [81–83]. A major peripheral site of high expression for this receptor is the GI tract [84–86]. Localization studies have suggested a distinct functional role for this protein in gut function and motility and compliment the reported actions of CRF and related endogenous peptides that have been examined in detail [63, 87, 88] and mimic the effects of stress on gastric and colonic motility [89, 90]. In fact, the concept of stress-induced pathophysiology in the gut and the role of the CRF receptors in mediating this pathology is currently generating a great deal of interest for drug discovery. The potential utility of CRF receptor antagonists for GI stress disorders such as irritable bowel syndrome have been recently reviewed [91–94].

The localization and distribution of the CRF₂ receptor are quite different and more varied within the CNS and across species than the CRF₁ receptor. Unlike the similarities in CNS distribution of the CRF₁ receptor between species, the CRF₂ receptor exhibits more species differences in the localization of receptor subtypes between rodent, nonhuman primate, and human tissues. Within the rat brain, CRF_{2(a)} receptor expression is generally confined to subcortical structures, including the lateral septal region, the bed nucleus of the stria terminalis, the amygdaloid area, and the olfactory bulb [77], whereas in higher order species such as human and nonhuman primates the CRF_{2(a)} receptor is distributed in high densities across the cortical regions as well as the brain structures described in rodents [77]. While the role of the CRF₂ receptor in anxiety disorders still requires some clarification, some recent studies have begun to demonstrate that at least the CRF_{2(a)} receptor may well facilitate the manifestation of anxiety. The lateral septum, by virtue of widespread reciprocal connections throughout the brain, is implicated in a variety of physiological processes playing a central role in classical limbic circuitry and thus potentially integrating a variety of emotional conditions including fear and aggression. In fact, it has recently been demonstrated that acute antagonism of the CRF₂ receptor in the lateral septum using the nonselective peptide antagonists produced a specific reduction in stress-induced behavior. This effect was not produced by the administration of selective CRF₁ receptor antagonists into this brain region [95]. In addition, it has been shown that selective peptide antagonists for the CRF₂ receptor administered intracerebroventricularly in mice were able to attenuate CRF or UCN2-induced increases in acoustic startle, suggesting in this model that the CRF₂ receptor may participate in the progression of the anxiety response [96]. In addition, CRF₂ receptor knockout studies have demonstrated that mice lacking the

CRF₂ receptor gene exhibit anxiety-like effects as well as a hypersensitivity to stress, further suggesting a role for this subtype in the overall response to stress [97]. Key to the understanding of the role of this receptor in anxiety or depression, of course, will be the development of selective nonpeptide receptor antagonists and their evaluation in primate models of disease. With these tools, a solid understanding of the role of this receptor subtype can be defined and the CRF_{2(a)} receptor may yet represent an additional target for the development of novel anxiolytics.

The CRF_{2(b)} splice form is localized primarily on nonneuronal elements, such as the choroid plexus of the ventricular system and cerebral arterioles. In the periphery, CRF_{2(b)} mRNA is expressed at high levels in both cardiac and skeletal muscle with lower levels evident in both lung and intestine [77, 98]. Most recently the CRF_{2(b)} receptor has been localized in the human cardiovascular system where the ligands UCN2 and UCN3 have been shown to cause vasodilation [99, 100]. Interestingly, these effects, if shown to be able to counteract the pressor effects of centrally mediated CRF₁ receptor effects of CRF and urocortin, may define a tight regulation of stress-induced cardiovascular changes [100]. The CRF_{2(c)} isoform has yet to be identified in the rodent; however, reverse transcriptase polymerase chain reaction (RT-PCR) analysis of human brain mRNA demonstrated expression in septum, amygdala, hippocampus, and frontal cortex [70]. A full characterization of the CRF_{2(c)} subtype and the role it may play in physiology or pathophysiology still remains to be determined. As with the CRF₁ receptor, the CRF₂ receptor is also highly expressed and localized to the GI tract [56, 84] and there have been recent reports demonstrating clear efficacy of the selective CRF₂ agonists UCN2 and UCN3 on GI motility and pain responses [101], further suggesting that this receptor subtype may be involved in the manifestation of stress- or anxiety-induced pathophysiology.

5.3 PEPTIDE LIGAND PHARMACOLOGY OF CRF₁ AND CRF₂ RECEPTORS

As defined above, the CRF receptor and ligand system is unusual in that the receptors are activated by multiple endogenous ligands (CRF, UCN1, UCN2, and UCN3 [42, 71, 102]). The receptors have been shown to couple to multiple G proteins and intracellular signaling pathways [103, 104] (reviewed in [105, 106]), but in general the most common signaling pathway is stimulation of cAMP production through activation of the G_s G protein. Peptide selectivity for the receptors has been assessed by measuring stimulation of cAMP accumulation. For the mammalian receptors overexpressed in cultured cell systems, CRF itself is moderately selective for CRF₁ over CRF₂ receptors [36, 37, 41, 65, 107–110], UCN1 is nonselective for the two subtypes [39–41, 108], and UCN2 and UCN3 are selective for the CRF₂ over the CRF₁ receptor [39–41, 109]. Interestingly, these peptides do not demonstrate any appreciable differences in activation potency when assessed at the three splice variants of the CRF₂ receptor (CRF_{2(a)}, CRF_{2(b)}, and CRF_{2(c)}) [70, 111]. A similar CRF₁/CRF₂ receptor selectivity profile has been demonstrated for receptors expressed endogenously (i.e., at normal physiological levels) in tissues and receptor-expressing cell lines [39, 41, 112–115]. For example, the pharmacological rank-order profile of peptide agonists at CRF₁ receptors in the AtT20 mouse corticotrope tumor cell line and CRF_{2(b)} receptors in A7r5 rat smooth muscle cells is similar to that in

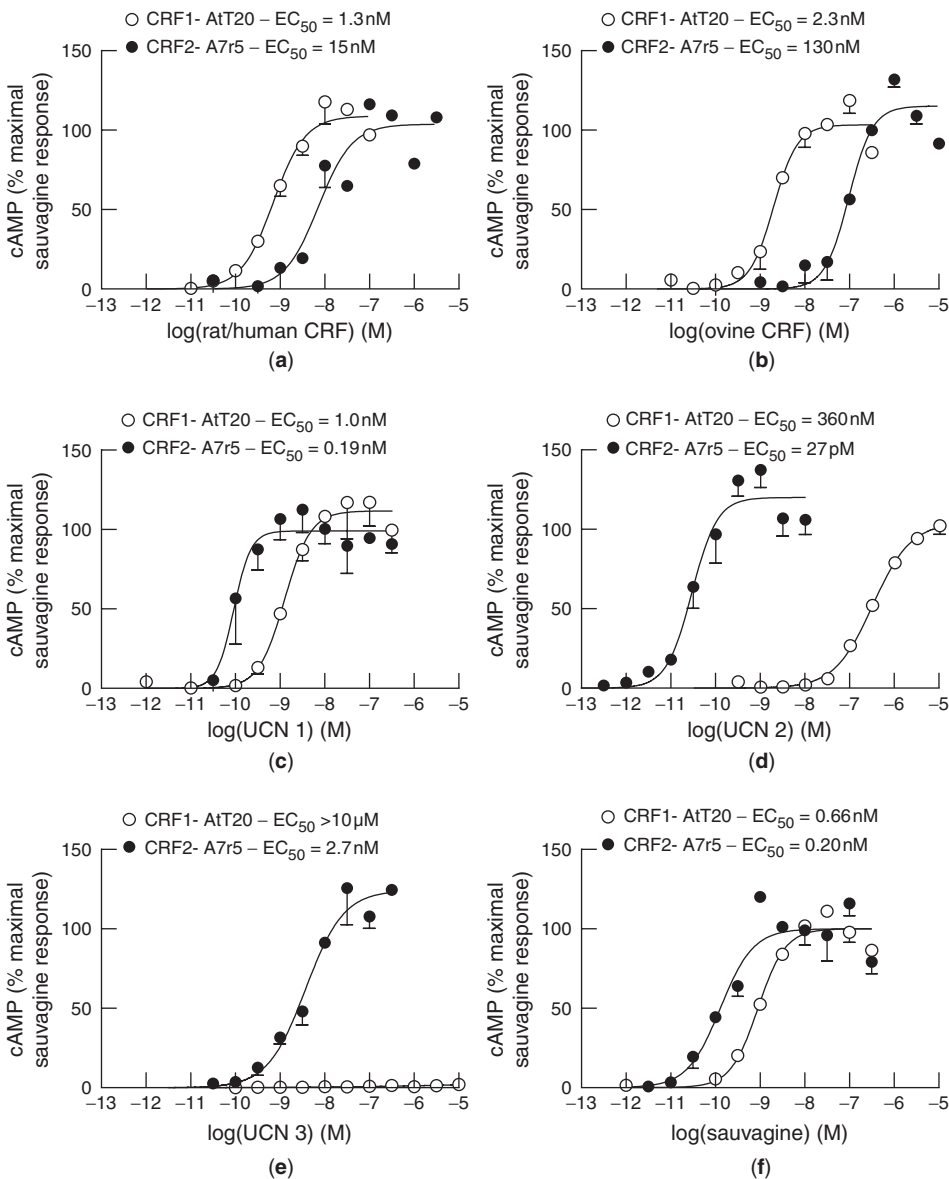


Figure 5.2 Stimulation of cAMP accumulation via CRF receptors expressed endogenously in model cell lines by CRF-related peptides. cAMP accumulation was measured in the mouse AtT20 corticotrope cell line, which expresses CRF₁, and the rat A7r5 aortic smooth muscle cell line, expressing CRF_{2(b)}. EC_{50} values are the mean of three experiments. (Data compiled from [116].)

heterologously expressed receptor systems (Fig. 5.2) [116]. This validation is important because receptor overexpression can distort pharmacological behavior of agonist ligands, for example resulting in enhancement of agonist potency [reduction of median effective concentration (EC_{50})] or enhancement of efficacy (enhanced E_{\max}).

As described above, synthetic peptide antagonists have been developed, by N-terminal truncation and amino acid substitution of the endogenous peptide agonists [57, 59–61, 109, 117, 118]. N-terminal truncation of CRF [57, 109], UCN1 [119], UCN2 [109], and sauvagine [60, 61] results in a loss of receptor activation but retention of detectable receptor binding. Certain critical amino acid substitutions of these truncated peptides increase affinity for one or both CRF receptors [57, 59–61]. For example, the nonselective antagonist astressin is a CRF(12–41) fragment with substitutions designed to enhance affinity (L-Phe¹¹ to D-Phe¹¹) and a lactam between residues 30 and 33 that stabilizes α -helical structure in the C-terminal portion of the peptide [61]. The extent of α helicity correlates well with the antagonist potency [57, 117]. Recently even shorter C-terminal analogs of astressin (12 amino acids, residues 30–41) have been reported that possess comparable potency to the parent peptide [118]. N-terminal truncation of sauvagine together with certain substitutions yielded the CRF₂-selective antagonists antisauvagine-30 [60] and astressin₂-B [61]. In addition to the truncated forms of agonists that yield antagonists, numerous peptide chimeras have also been developed by combining sequence fragments of the different endogenous peptides. Examples include cortagine, an agonist selective for the CRF₁ receptor over the CRF₂ receptor [110].

Peptide ligand binding to CRF₁ and CRF₂ receptors has been measured using radiolabeled peptides. Both receptor subtypes are readily labeled by [¹²⁵I]sauvagine [65, 116, 120, 121], an agonist, and [¹²⁵I]astressin [121, 122], an antagonist. Other radioligands include radiolabeled UCN1 [122–124] for CRF₁ and CRF₂ receptors, [¹²⁵I]oCRF for selectively labeling CRF₁ [30, 124, 125], and [¹²⁵I]antisauvagine-30 for selectively labeling CRF₂ [126]. Ligand binding affinity for G-protein-coupled receptors is dependent on the conformational state of the receptor. In general, agonists bind with higher affinity to the G-protein-coupled state (RG state) than the uncoupled receptor (R state), whereas antagonist binding does not discriminate between these states [127, 128]. Taking this differential binding pharmacology into account can be important for identifying receptor subtypes in tissues and for understanding ligand binding mechanisms (see below). For CRF receptors the RG state can be selectively labeled using a radiolabeled agonist [30, 65, 116, 120, 121, 124, 125] such as [¹²⁵I]sauvagine [116, 121]. The R state can be generated using guanosine triphosphate (GTP) or its nonhydrolyzable analogs (such as GTP γ S). These guanine nucleotides bind the α subunit of heterotrimeric G proteins, leading to the breakdown of the receptor–G protein complex [129]. The resulting R state of CRF receptors can be labeled by the antagonist [¹²⁵I]astressin [116, 121, 122]. For the CRF₁ receptor, peptide agonists bind with much higher affinity to the RG state than to the R state (Fig. 5.3, Table 5.1) and vary from 77-fold for UCN1 to 690-fold for oCRF [121]. This finding suggests uncoupling the G protein from its receptor substantially reduces binding of peptide agonists to the CRF₁ receptor, implying agonism results at least in part from ligand discriminating the RG state over the R state. By contrast, peptide agonist binding to the CRF₂ receptor is much less sensitive to the conformational state of the receptor with a change of 1.2-fold higher affinity for RG for UCN1 to a 6.5-fold difference for UCN2 (Fig. 5.3, Table 5.1 [116]). This finding suggests that for the CRF₂ receptor uncoupling receptor from G protein does not strongly affect peptide agonist binding, a hypothesis supported by the only slight reduction of [¹²⁵I]sauvagine binding produced by GTP γ S (Fig. 5.3i) [65, 116, 120, 126]. Peptide antagonist affinity for both receptors was unaffected by

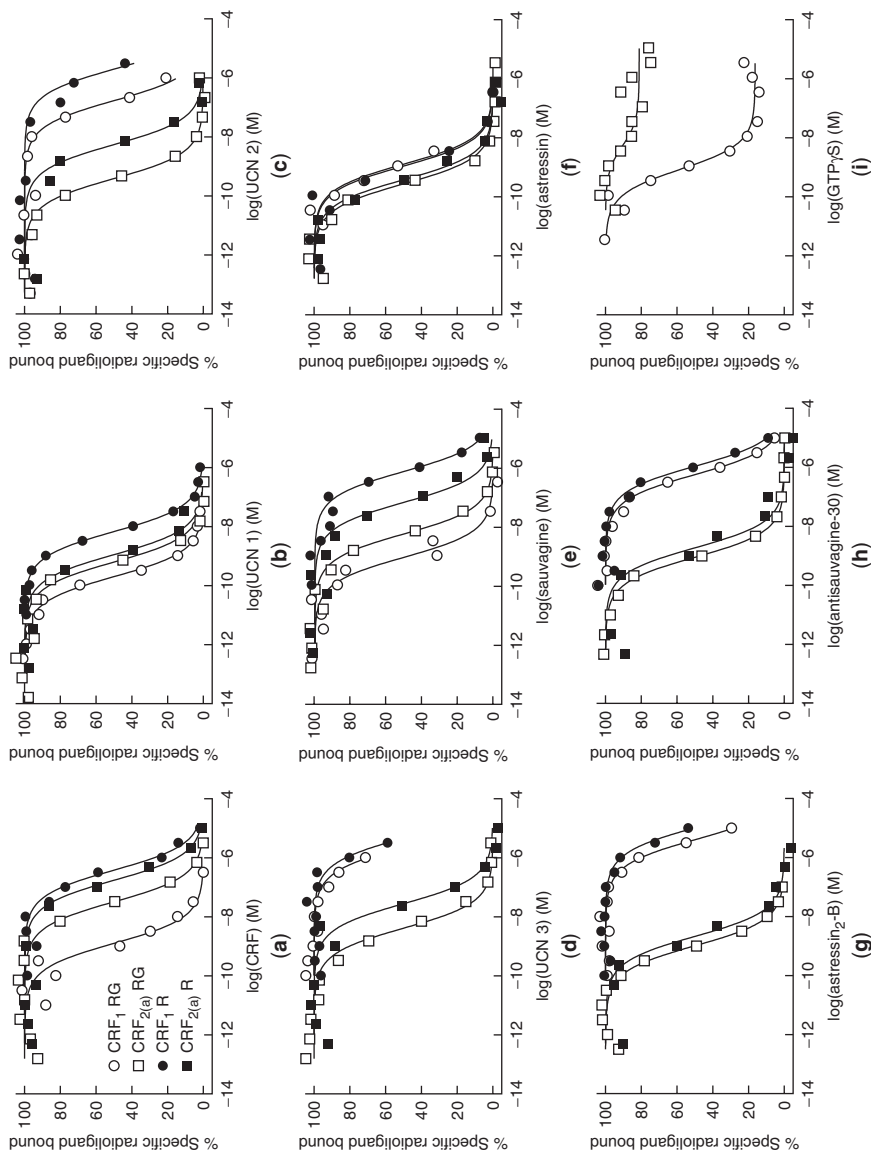


Figure 5.3 Peptide ligand affinity for different receptor states of CRF receptors. Peptide ligand affinity can be dependent on the state of the receptor. Peptide affinity for the G-protein-coupled state (RG state) of the human CRF_1 and $\text{CRF}_{2(a)}$ receptors can be measured by competition against a radiolabeled peptide agonist ($[^{125}\text{I}]\text{sauvagine}$). Peptide affinity for the receptor uncoupled from G protein (R state) was measured in competition versus a radiolabeled antagonist peptide ($[^{125}\text{I}]\text{astressin}$) in the presence of $\text{GTP}\gamma\text{S}$. This guanine nucleotide binds the α subunit of heterotrimeric G proteins, leading to the breakdown of the receptor-G protein complex. Data for the human CRF_1 receptor are for this receptor in membranes prepared from LtK^- mouse fibroblasts [121]. Data for the human $\text{CRF}_{2(a)}$ receptor are for this receptor in Chinese hamster ovary cell membranes. (Data compiled from [116, 121].)

TABLE 5.1 Peptide Ligand Affinity (nM) for R and RG States of Human CRF₁ and CRF_{2(a)} Receptors^{a,b}

Peptide Ligand	RG State		R State	
	CRF ₁ K _i (nM)	CRF ₂ K _i (nM)	CRF ₁ K _i (nM)	CRF ₂ K _i (nM)
r/hCRF	0.30	19	270	38
oCRF	0.29	150	200	320
hUCN1	0.039	0.27	3.0	0.33
hUCN2	130	0.17	640	1.1
hUCN3	> 1000	1.3	> 1000	6.6
Sauvagine	0.45	2.6	230	11
Astressin	0.43	0.15	0.67	0.11
Antisauvagine-30	150	0.29	340	0.40
Astressin ₂ -B	950	0.49	4200	0.57

^aSource: Data compiled from [116, 121].

^bPeptide ligand affinity for G-protein-coupled (RG) receptor state was measured in competition versus a radiolabeled agonist peptide ([¹²⁵I]sauvagine) for human CRF₁ or CRF_{2(a)} receptors expressed in mammalian cells (L + k⁺ cells [121] and CHO cells, respectively [116]). Peptide affinity for the receptor uncoupled from G protein (R state) was measured in competition versus a radiolabeled antagonist peptide ([¹²⁵I]astressin) in the presence of GTPγS. This guanine nucleotide binds the α subunit of heterotrimeric G proteins, leading to the breakdown of the receptor–G protein complex.

the conformational state of the receptor (Figs. 5.3f–h, Table 5.1 [108, 116, 121, 122, 124, 126]), implying antagonism results from a lack of discrimination between RG and R states.

Peptide ligand selectivity for binding one CRF receptor over the other has historically been determined using radiolabeled agonists [65, 108, 116, 120, 121, 123], particularly [¹²⁵I]sauvagine. Under these conditions, affinity estimates for receptors are likely those for the RG state of CRF₁ and CRF₂ receptors. Selectivity in binding the RG state approximately correlates with selectivity in functional cAMP accumulation experiments: in the binding assays, r/hCRF is selective for CRF₁ over CRF₂ (63-fold for cloned human CRF₁ and CRF_{2(a)} receptors; Table 5.1, Fig. 5.3a); oCRF is more CRF₁ selective (520-fold, Table 5.1); UCN1 and sauvagine are nonselective (Table 5.1, Figs. 5.3b,e); UCN2 and UCN3 are strongly CRF₂ selective (760-fold and > 770-fold, respectively, Table 5.1, Figs. 5.3c,d); astressin is a nonselective antagonist (Table 5.1, Fig. 5.3f); and astressin₂-B and antisauvagine-30 are CRF₂-selective antagonists (510-fold and 1900-fold, respectively, Table 5.1, Figs. 5.3g,h) [39, 60, 108, 109, 116, 121, 122]. This selectivity profile has been demonstrated for CRF receptors expressed endogenously in tissues (e.g., cerebellum for CRF₁ [121] and olfactory bulb for CRF₂ [116]). A different selectivity profile is manifest for the uncoupled R state of the receptor. In this conformation, CRF peptides are no longer selective (Table 5.1, Fig. 5.3a) and UCN1 and sauvagine are slightly selective for CRF₂ over CRF₁ receptors (9.0-fold and 21-fold, respectively, Table 5.1, Figs. 5.3b,e) [116]. Therefore, in identifying the receptor subtype in tissues it is important to consider the nature of the conformational state labeled by the specific radioligand used. The use of agonist radioligands is recommended, enabling comparison with a wealth of historical data.

5.3.1 Mechanisms of Peptide Ligand Binding to CRF₁ and CRF₂ Receptors

The molecular mechanisms of peptide ligand binding to CRF receptors have been investigated using point-mutated receptors [130–139], chimeric receptors [116, 130–134, 138, 140, 141], receptor fragments [64, 119, 139, 140, 142–146], receptor–ligand crosslinking [136, 147], and nuclear magnetic resonance (NMR) chemical shift perturbation [139]. These studies have indicated a low-resolution binding orientation termed the “two-domain” model, also demonstrated for other class B GPCRs. In this mechanism, the carboxyl terminal portion of the peptides interacts with the N-terminal domain (N-domain) of the receptors, and the amino terminal portion of the peptide binds the receptors’ juxtamembrane domain (J domain, comprising the transmembrane helices and intervening loops). Binding determinants in both receptor domains have been identified using receptor mutants and chimeric receptors (Fig. 5.1). Peptide ligands interact with both N- and J-domain fragments expressed in isolation (Fig. 5.4). The carboxyl terminal fragments of CRF and sauvagine

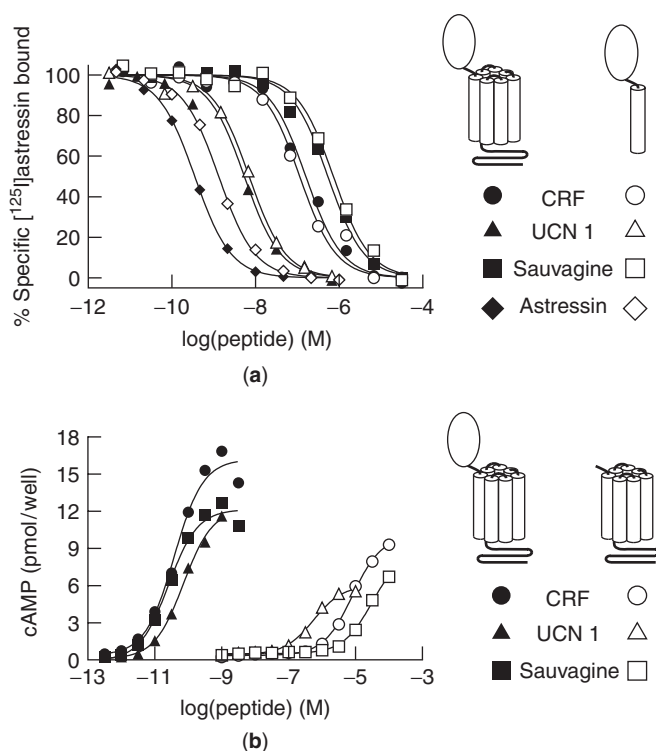


Figure 5.4 Interaction of peptide ligands with (a) N domain and (b) J domain of CRF₁ receptor. (a) Peptide ligand interaction with the N domain was measured by inhibition of [¹²⁵I]astressin binding to the N domain fused to the single-membrane-spanning α helix of the activinII-B receptor [140, 146]. Peptide affinity for the whole receptor was measured by inhibition of [¹²⁵I]astressin binding in the presence of GTP γ S to uncouple receptor from G protein. (b) Peptide ligand interaction with the J domain was measured by stimulation of cAMP accumulation via a J-domain fragment [146]. Note that peptide potency on the J domain is dramatically reduced compared with the whole receptor. The peptides did not affect cAMP accumulation in nontransfected cells [146]. (Data compiled from [146].)

(astressin and antisauvagine-30, respectively) bind with high affinity to N-domain fragments of CRF₁ and CRF₂ receptors [64, 140, 144–146] (Fig. 5.4a). Modifying the carboxyl terminal residue of CRF by deamidation eliminates detectable binding to the N-domain fragment of the CRF₁ receptor but does not affect interaction with the J-domain fragment [146]. Tethering the N-terminal 16 amino acids of CRF to the J domain of the CRF₁ receptor results in receptor activation [143]. Receptor–ligand crosslinking, utilizing the CRF₁ receptor and peptides with photoactivatable side chains, indicated residue 16 of sauvagine crosslinks L257 in the second extracellular loop [136]; the extreme N-terminus and residue 12 of UCN1 bind the second extracellular loop; and residues 35 and 40 of UCN1 bind the C-terminal region of the N domain [147].

The molecular mechanism has been evaluated to a higher resolution for the interaction of astressin with the N domain, utilizing structural data for peptide and the N domain [139]. NMR, circular dichroism, and other analytical studies of CRF-related peptide structure indicate transient-to-stable formation of α helix within the C-terminal region [57, 59, 117, 118, 148, 149], depending on the experimental conditions and peptide studied. An NMR structure of a soluble N domain of the mouse CRF_{2(b)} receptor was recently reported [139]. A central core contains a salt-bridge sandwiched between aromatic side chains surrounded by conserved residues. Two antiparallel β sheets are interconnected by the core. Loop regions of poorly resolved structure are adjacent to the structured core region. The tertiary structure is stabilized by three disulfide bonds (C45–C70, C60–C103, and C84–C118 for the mouse CRF₂ receptor). These three cysteines are conserved within CRF receptors and other class B GPCRs [64, 139, 144, 150]. NMR chemical-shift perturbation within the N domain upon binding astressin [139] indicates residues in and around the structured core are direct or indirect binding determinants (I67, G68, T69, G90, I91, K92, N94, A99, Y100, E102, R112, V113, N114, Y115, and S116). These residues are highly conserved within CRF receptor subtypes and species variants, suggesting a common molecular mechanism of peptide ligand binding to the N domain [139]. Mutation of I67 and R112 of the CRF_{2(b)} receptor reduces affinity of astressin 120- and 6.5-fold, respectively [139]. Mutation of residues analogous to K92 and A99 of the CRF_{2(b)} receptor in the CRF₁ receptor also reduces peptide ligand affinity [132]. These data can be rationalized by a molecular model of astressin interaction with the CRF_{2(b)} N domain, in which the carboxyl terminal portion of astressin binds as an α helix to a broad, slightly concave surface within the structured core region [139]. Proposed interactions between an astressin and the mouse CRF_{2(b)} receptor include electrostatic interaction between E39 and R112 and between K35 and E119 and hydrophobic interaction between L37 and Y115 and between I41 and P120 [139].

Less is known about the molecular interactions of the amino-terminal portion of the peptide and the J domain of the receptor. The solution structure of the amino-terminal portion of the peptide is not well characterized, and nothing is presently known of the receptor-bound structure of this region of the peptide. In addition the structure of the J domain of CRF receptors has not been determined. The transmembrane helices of this region have been modeled by homology using the crystal structure of bovine rhodopsin as a template, but the predictive utility of these models might be limited owing to the very low amino acid sequence homology between CRF receptors (class B GPCRs) and rhodopsin (a class A GPCR) [139]. The

only obvious common feature between class A and B GPCRs is the potential disulfide bond between transmembrane type 3 (TM3) and extracellular loop 2, although evolutionary trace analysis has suggested a small number of other common hydrophobic amino acid residues within the transmembrane domains [151, 152]. Peptide binding determinants within the J domain are located within the predicted extracellular loops and toward the predicted extracellular ends of transmembrane helices (Fig. 5.1). These results suggest the amino-terminal portion of peptide ligand binds to the extracellular face of the J domain of CRF receptors, an arrangement that has also been suggested for other class B GPCRs [150]. Interestingly, circumstantial evidence suggests N terminally truncated CRF and sauvagine analogs can interact with the J domain of the receptors. Astressin, a 12–41 residue analog of CRF, blocks activation of a CRF₁ J-domain fragment by CRF, albeit at high concentrations [146]. Additionally, this peptide binds with 10-fold higher affinity to the whole CRF₁ receptor than an N-domain receptor, suggesting additional binding determinants within the J domain [146]. Shorter C-terminal CRF analogs (residues 30–41, peptides 19 and 20 of [118]) bind with similar affinity to the whole receptor and the N domain, suggesting residues within the 12–29 region of astressin bind the J domain of the CRF₁ receptor [153]. Finally, analysis of chimeric CRF₁/CRF₂ receptors indicated that the J domain acts as a selectivity determinant for CRF₂-selective antisauvagine-30 and astressin₂-B (11–40 analogs of sauvagine), suggesting these peptides interact with the J domain of the CRF₂ receptor [116]. Taken together these findings suggest peptide antagonists can bind the J domain of CRF₁ and CRF₂ receptors, possibly through interactions with amino-terminal residues of the peptides.

The J domain of the CRF₁ receptor is involved in ligand binding and conformational changes of the receptor that lead to receptor activation and desensitization. Peptide agonists stimulate cAMP accumulation via a J-domain fragment (Fig. 5.4b) [146] and the amino-terminal portion of CRF activates a J-domain fragment when tethered to this receptor [143]. In addition, full-length peptide agonist binding to the CRF₁ receptor is markedly reduced by GTP γ S, whereas residue 12–41 analogs of CRF (e.g., astressin) are unaffected (see above). This finding suggests interactions of residues within the 1–11 sequence with the J domain are sensitive to G-protein coupling to the receptor. Peptides that appear to only bind the N domain (peptides 19 and 20 in [118]) do not activate the CRF₁ receptor, suggesting ligand interaction with the N domain is not required for receptor activation. These short fragments also fail to affect CRF₁ receptor internalization, whereas full-length CRF robustly stimulates internalization [153]. This result provides circumstantial evidence that ligand interaction with the J domain but not the N domain stimulates CRF₁ receptor internalization. Interestingly, astressin also stimulates CRF₁ receptor internalization, an unusual effect of an antagonist ligand [153]. This effect is mediated through receptor interaction of the 12–29 region of the peptide [153], likely through binding the J domain (see above). The receptor conformation mediating astressin-induced receptor internalization appears distinct from that for CRF; the latter ligand promotes receptor phosphorylation and arrestin recruitment to the receptor, whereas the former ligand does not [153].

The strength of peptide agonist interaction for each of the N and J domains has been quantified for the CRF₁ receptor using N- and J-domain fragments. UCN1 binds with high affinity (approximately 5 nM) to the CRF₁ N domain expressed in isolation as a membrane-proximal protein (expressed as a fusion with the single

membrane-spanning α helix of the activinII-B receptor) [64, 140, 146]. CRF and sauvagine bind with lower affinity to the N domain (approximately 50 and 500 nM, respectively [64, 140, 146]). Similar data were obtained with a soluble CRF₁ N-domain fragment [64, 119, 144, 145]. Peptide ligand affinity for the membrane-anchored N-domain fragment has been compared with that for the whole CRF₁ receptor, enabling quantification of the contribution of the N domain to overall ligand affinity. In this comparison the G-protein-uncoupled (R) state of the full-length CRF₁ receptor was used, since the N domain on the extracellular membrane surface is incapable of coupling to G protein on the intracellular membrane surface. For the CRF₁ receptor these experiments demonstrated that peptide agonist affinity for the N domain is only slightly stronger (less than twofold) on the full-length, G-protein-uncoupled CRF₁ receptor than on the membrane-anchored N domain fragment, indicating the N domain contributes almost all the ligand binding energy at the R state [140, 144, 145]. Direct peptide binding to the J domain of the CRF₁ receptor is extremely weak—on the isolated J-domain fragment, peptide agonist EC₅₀ for stimulating cAMP production was four to six orders of magnitude higher than EC₅₀ for the whole receptor (Fig. 5.4b) [146]. Taken together these findings suggest a sequential mass action binding model for agonist peptides [146] in which peptide first binds the N domain of the CRF₁ receptor with moderate to high affinity (depending on the peptide agonist) (Fig. 5.5a). This interaction provides an “affinity trap” that enormously increases the local concentration of peptide in the vicinity of the J domain, allowing the weak J domain interaction to occur (Fig. 5.5a). This concentrating effect of N-domain binding enables significant ligand interaction to occur with the J domain at physiological levels of ligand. Ligand interaction with the J domain promotes receptor activation and stimulation of G-protein coupling leading to intracellular signaling (Fig. 5.5a). Reciprocally, G-protein coupling to the CRF₁ receptor increases the affinity of peptide agonists by 49- to 690-fold [121], likely through increasing peptide affinity for the J domain [146]. Taken together, these findings suggest a quantitative model for the CRF₁ receptor in which (1) peptide agonists bind with moderate to high affinity for the N domain (100 nM for CRF); (2) peptide binding to the J domain weakly stabilizes binding at the R state of the receptor (<2-fold for CRF); and (3) peptide binding to the J domain is strongly stabilized by G-protein coupling to the receptor (> 100-fold for CRF).

Similar techniques have been applied to estimate peptide agonist affinity for the CRF₂ receptor. UCN1 and UCN2 bind with high affinity to a membrane-anchored CRF_{2(b)} receptor N domain (5.4 and 16 nM, respectively), whereas UCN3, CRF, and sauvagine are less potent (> 200, 79, and > 200 nM, respectively) [145]. Similar data were obtained with a soluble CRF_{2(b)} receptor fragment [145]. Peptide agonist affinity for the whole CRF_{2(b)} receptor (R state) was significantly higher than affinity for the isolated J domain (from 2.9-fold for CRF to 33-fold for UCN2) [145], suggesting an appreciable contribution of the J domain to peptide agonist affinity at the R state (in contrast to the CRF₁ receptor for which the J domain contributed minimally to peptide agonist affinity at the R state). G-protein coupling to the CRF_{2(a)} or CRF_{2(b)} receptor only weakly stabilizes peptide agonist binding (e.g., 1.2- to 6.5-fold for CRF_{2(a)}). Within the context of the two-domain model, these results imply that, first, peptide agonists bind with moderate to high affinity to the N domain of the CRF₂ receptor (16 nM for UCN2); second, binding is moderately stabilized through peptide interaction with the J domain of the R state (33-fold for UCN2); and third,

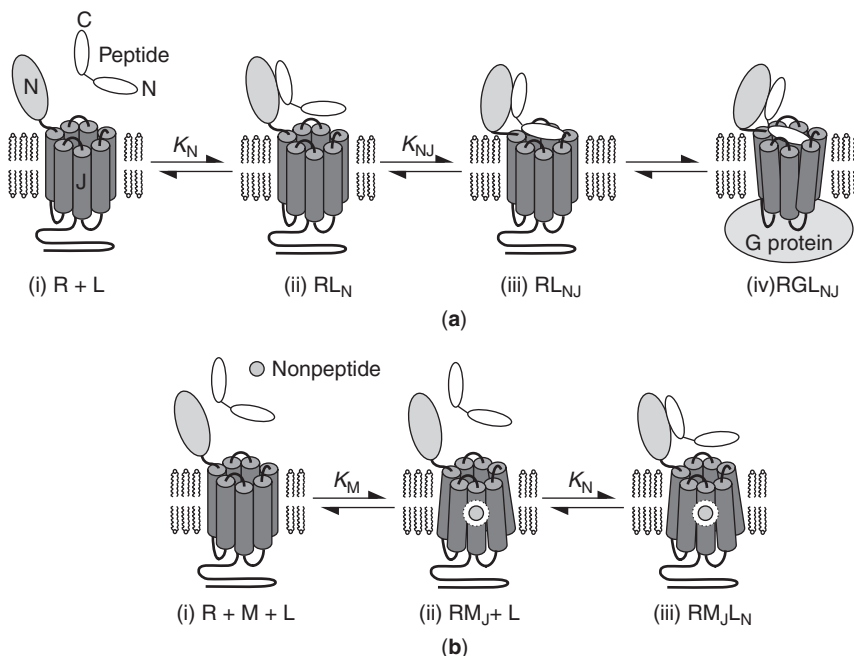


Figure 5.5 Binding models of peptide and nonpeptide ligand interaction with CRF receptors. (See [146] for a detailed description of these models.) (a) Binding model of peptide ligand interaction with CRF_1 and CRF_2 receptors. (i) The C-terminal region of the peptide (L) binds the N domain of the receptor (R) forming RL_N . (ii) This interaction enormously increases the local concentration of the N-terminal peptide region in the vicinity of the J domain, allowing (iii) their weak interaction to occur, forming RL_{NJ} . (iv) J-domain peptide binding increases receptor interaction with G protein, and reciprocally G-protein binding enhances J-domain affinity for peptide ligand. (b) Nonpeptide binding and antagonism model for CRF_1 receptor. (i) Nonpeptide ligand (M, small circle) binds within the J domain, forming RM_J . (ii) This interaction causes a change within the CRF_1 receptor that impedes peptide binding to its sites on the J domain. By blocking peptide–J domain interaction, the nonpeptide antagonist blocks peptide-stimulated receptor signaling because peptide–J domain interaction is required for G-protein activation. (iii) Nonpeptide ligand binding to the J domain does not prevent peptide interaction with the N domain, so the RM_JL_N complex can form. (Reproduced with permission from *Drug Discovery Today* [150].)

G-protein coupling to the receptor weakly enhances peptide agonist binding (6.5-fold for UCN2). Compared with the CRF_1 receptor (see above), the J domain appears to provide more binding energy for the CRF_2 receptor (at the R state), whereas G-protein coupling stabilizes binding less. The molecular basis of this difference between the two receptors remains to be determined.

Finally the strength of peptide antagonist interaction for N and J domains has been assessed. For both CRF_1 and $CRF_{2(b)}$ receptors astressin binds with high affinity to N-domain fragments (approximately 1 nM). Surprisingly, astressin affinity for the whole receptor is an order-of-magnitude higher for both CRF_1 and $CRF_{2(b)}$ receptors [145, 146], suggesting astressin binding is appreciably stabilized through interaction with the J domain. In addition, the J domain is a selectivity determinant of the CRF_2 -selective antagonists antisauvagine-30 and astressin₂-B, shown using

chimeric CRF₁/CRF₂ receptors [116]. Peptide antagonist binding is unaffected by receptor–G protein interaction [116, 121, 122]. These findings suggest peptide antagonists bind with high affinity to the N domain, with binding moderately stabilized through interaction with the J domain and with G-protein binding to the receptor having no effect.

5.4 NONPEPTIDE LIGANDS FOR CRF₁ RECEPTORS

Antagonism of central CRF₁ receptors has been rationalized as a potential next-generation treatment for anxiety and depression [154–157]. Since peptide antagonists generally possess poor pharmacokinetics (poor oral bioavailability, rapid clearance, and minimal brain penetration) and lack CRF₁ receptor selectivity, attempts have been made to develop small, orally bioavailable, brain-penetrating, CRF₁-selective nonpeptide antagonists. Numerous low-molecular-weight ligands have been developed that potently bind and antagonize the CRF₁ receptor (examples in Fig. 5.6; reviewed in detail in [156, 158, 159]). The large majority of these ligands comprise a central heterocyclic core (examples include the monocyclic SSR125543A [160, 161], the bicyclic antalarmin [162], and tricyclic NBI 35965 [163, 164]; Fig. 5.6), a “top” alkyl or branched alkyl side chain, and a “bottom” substituted aromatic ring. Regardless of the core composition, there is an essential requirement for a hydrogen bond acceptor, separated from the bottom aromatic group by a one-atom linker (e.g., SSR125543A) or by a two-atom linker (e.g., antalarmin) (see Fig. 5.6). Most, if not all, known nonpeptide antagonists display considerable selectivity for the CRF₁ receptor over the CRF₂ receptor (for a comprehensive review on structures see [156, 157]). This selectivity, combined with reasonable pharmacokinetics, has rendered compounds such as these exceptionally useful for evaluating the physiological and potential therapeutic roles of the CRF₁ receptor in animal models and, as described below, has been initially examined in an open label phase IIA clinical trial in major depressive disorder [165].

5.4.1 Mechanism of Nonpeptide Ligand Interaction with CRF₁ Receptor

The mechanism of nonpeptide ligand binding to the CRF₁ receptor has been investigated using receptor fragments [143, 146], receptor chimeras and mutagenesis [130], and quantitative analysis of ligand binding data [163, 166]. Nonpeptide antagonists bind the J domain, predominantly if not exclusively: Nonpeptide radioligand affinity for a J-domain fragment is not significantly different from that for the whole receptor and nonpeptide antagonist fully blocks peptide-stimulated cAMP accumulation via the J-domain fragment [146]. Constitutive activation of a J-domain fragment tethered to the N-terminal region of CRF is blocked by the nonpeptide antagonist antalarmin [143]. CRF₁/CRF₂ receptor chimeras identified TM3 and TM5 as selectivity determinants for selective nonpeptide antagonist with the CRF₁ receptor [130]. Exchange of two residues of the CRF₁ receptor for the corresponding residues of the CRF₂ receptor resulted in reduced nonpeptide ligand binding (H199 V in TM3 and M276I in TM5; Fig. 5.1) [130].

Nonpeptide antagonist likely binds regions within the J domain that are distinct from those for peptide ligand. The two mutations of the CRF₁ receptor implicated in

nonpeptide ligand binding did not affect peptide ligand binding [130]. In addition these determinants are within the predicted transmembrane bundle (Fig. 5.1), whereas peptide ligand binding determinants have been identified principally in the extracellular loop regions of the J domain (Fig. 5.1). In addition, nonpeptide versus peptide ligand binding data display diagnostic features implying nonpeptide ligand allosterically regulates peptide ligand binding, and vice versa: Nonpeptide ligands modulate peptide radioligand dissociation from the CRF₁ receptor, and peptide ligands accelerate nonpeptide radioligand dissociation [163]. In addition peptide ligands only partially inhibit nonpeptide radioligand binding at equilibrium [163, 166]. An interesting difference was observed between the allosteric effect at G-protein-coupled and G-protein-uncoupled CRF₁ receptor states. At the R state, the allosteric effect between peptide and nonpeptide ligand binding was slight. NBI 35965 binding to the receptor reduced sauvagine affinity by only 3.0-fold. In other words, sauvagine affinity for the NBI 35965-bound receptor was 690 nM compared with 230 nM for the free receptor). In contrast, at the RG state NBI 35965 binding reduced sauvagine affinity by 180-fold.

All of these observations can be explained by the model presented in Fig 5.5b and have been described in detail [146]. In this model, nonpeptide ligand binds to sites within the transmembrane region of the J domain, distinct from the sites bound by peptide ligand (Fig. 5.5b). Nonpeptide ligand binding to this region produces a change in the receptor that impedes peptide binding to its sites on the J domain. This allosteric effect blocks peptide-stimulated signaling, since J domain interaction is required for peptide agonism (see above). However, this allosteric effect does not affect peptide binding to the N domain of the receptor (Fig. 5.5b). This model can explain why the allosteric effect is greater at the RG state than at the R state. At the RG state the J domain contributes much more binding energy for peptide ligand interaction, so blocking this interaction with nonpeptide ligand substantially reduces peptide binding affinity. At the R state the J domain contributes much less peptide binding energy, so blocking this interaction only marginally reduces peptide binding. Overall this binding model retrospectively rationalizes nonpeptide-versus-peptide binding data, explains the antagonist action of the compounds, and could be prospectively useful in the future development of nonpeptide ligands.

5.5 POTENTIAL FOR THERAPEUTIC INTERVENTION IN ANXIETY AND DEPRESSION

During the course of examining the role of the CRF system in disorders such as anxiety and depression, a number of preclinical studies have suggested that anxiety-related disorders such as generalized anxiety disorder or panic disorder, while independent syndromes, share some clinical and biological characteristics with major depression. In addition to the circumstantial evidence presented above from clinical studies monitoring the regulation of the CRF system in various disease and treatment paradigms, there have been a great many experimental studies performed in animal models supporting the role of this system in the manifestation of anxiety- or depression-like behaviors. It is not surprising that the human disorders of anxiety and depression are difficult to model in animals. However, animal models have been widely used focusing on specific symptoms involving fear and/or fearful responses in

animals that are correlated to the inappropriate responses to mildly stressful stimuli exhibited in human anxiety. For example, the findings that CRF itself has central, behavioral and arousal properties that are characteristic of other anxiogenic compounds have been well documented preclinically and relate directly to the hyperarousal that defines anxiety disorders [167, 168]. In panic disorder, a role for CRF has been suggested by the observations again of a blunted ACTH response in these patients compared to normal individuals [169, 170]. The blunted ACTH response in panic disorder patients to exogenous CRF most likely reflects processes occurring above the level of the hypothalamus and related to a central hypersecretion of CRF. In a recent preclinical study, CRF-induced deficits in prepulse inhibition (PPI) in rats were reversed by pharmacological blockade with CRF receptor antagonists. The CRF-induced deficit in animals is similar to the disruption of PPI observed in patients with panic disorder where the CRF system may also be overactive [171]. Thus, by modeling some of the physiological characteristics of a disorder, it is possible to examine the mechanisms that may underlie a specific pathophysiology. There have been a number of excellent reviews detailing the myriad animal models that have been used to dissect the precise effects and regulation of CRF in stress-related behaviors [156, 172–175].

The majority, if not all, of the small-molecule CRF receptor antagonists that have been described are primarily selective for the CRF₁ receptor subtype. The few molecules that have appeared in the literature claiming CRF₂ selectivity have been at best weak inhibitors of function at the CRF₂ receptor subtype. The efforts to further develop these compounds have focused on improving their physicochemical and pharmacokinetic properties to maximize in vivo efficacy. Nonpeptide small molecules have demonstrated efficacy in a variety of rodent models of anxiety, including changes in exploratory behavior, a measure of innate fearfulness [160, 176, 177] social interaction (anxiety) stressful situations [177–181], conditioned fear responses [182–184], and acoustic startle responses [96, 185, 186]. In addition to the rodent studies, these molecules have also demonstrated efficacy in many nonhuman primate models of anxiety. For example, both antalarmin and DMP-904 (Fig. 5.6) reduced stereotypic fear responses and decreased measures of anxiety in the human intruder stress paradigm in rhesus monkey while, in addition, significantly attenuating HPA activation and the stress-induced increase in CSF CRF [187, 188]. Chronic oral administration of antalarmin in male rhesus monkeys was also demonstrated to attenuate the stress associated with social separation in these animals [189]. Finally, CRF₁ receptor antagonists have been demonstrated to block the stress-induced visceral hyperalgesia and colonic motility in rats [190], suggesting that these molecules may also have utility in stress- or anxiety-related GI disorders [91, 94]. A thorough evaluation of the current CRF₁ receptor antagonist small molecules and their potential utility in anxiety disorders has recently been published [191].

Despite the heroic efforts expended by the pharmaceutical industry in discovering novel chemical structures that interact with the CRF receptor system, there has only been one single clinical study published in the literature describing the effects of a CRF₁ receptor antagonist in depression. In this open label non-placebo-controlled phase IIA study conducted at the Max Planck Institute of Psychiatry, Munich, the pyrazolopyrimidine R121919 (NBI 30775) was found to be well tolerated in 20 patients following a 30-day, dose escalation paradigm up to 80 mg. This compound demonstrated a significant reduction in both the Hamilton depression and anxiety

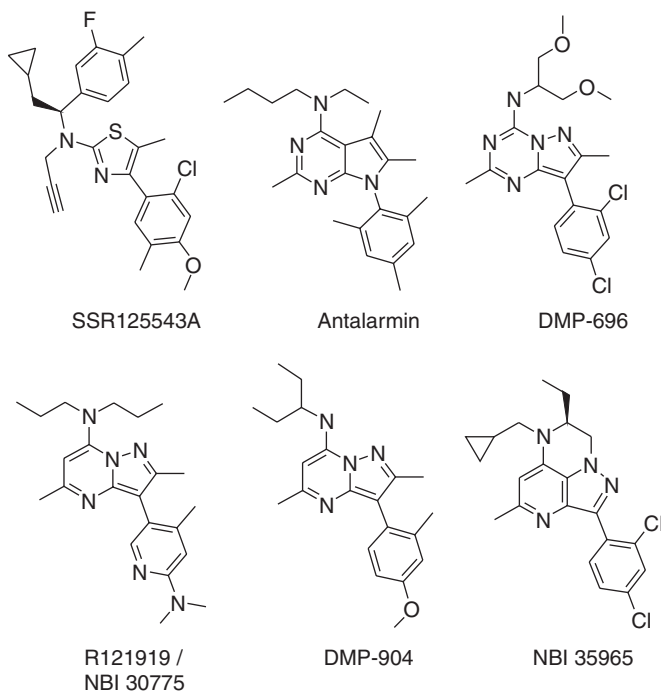


Figure 5.6 Chemical structure of nonpeptide antagonists for CRF₁ receptor. Detailed reviews of CRF₁ nonpeptide ligands can be found in [156, 158, 159].

scales HAM-D and HAM-A across the treatment period without affecting either basal HPA activity or significant blunting of an exogenously administered CRF-induced ACTH response [165]. Furthermore, this molecule was found to improve sleep-EEG patterns in both human and rat [192, 193] and in a very recent study demonstrated that this treatment did not affect weight gain or plasma leptin levels in this group of depressed individuals [194]. Although in this small open label trial the compound did not demonstrate any untoward effects in the patient population [165, 194, 195], this particular compound caused some reversible liver enzyme elevations that precluded further clinical development. Nevertheless, this initial study has provided significant support for the use of CRF₁ receptor antagonists in the treatment of depression- or anxiety-related disorders. More importantly, this study suggested that it would be possible to separate the central efficacy of CRF₁ receptor blockade from the potential peripheral side effects of rendering the HPA axis unresponsive [195].

5.6 CONCLUSIONS

The discovery of selective CRF₁ receptor antagonists has completed the first steps required in the development of novel therapeutics for stress-related disease. These newly characterized tools have in turn led to the refinement and expansion of the initial hypotheses and have identified a tangible goal of producing selective molecules

that will target specific CRF-mediated behavior and physiology. While a preclinical “proof of principle” has been established whereby blockade of this system has demonstrated clear and precise functional benefits, the definitive studies in human disease have not yet been possible. Nonetheless, with the importance of this system in physiology and pathophysiology and the global efforts of academic and pharmaceutical researchers, there is little doubt that safe and selective molecules for this family of receptors will soon become available and eventually lead to some exciting novel therapeutic opportunities for neuropsychiatric and stress-related diseases. The CRF system is positioned to offer an alternative treatment option, and should the hypothesis that the cause of these disorders is manifested through an underlying overactivity of the stress system be confirmed, CRF receptor antagonists may well be the beginning of the next generation of antidepressant therapies with a novel mechanism of action.

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6

NEUROBIOLOGY AND PHARMACOTHERAPY OF OBSESSIVE-COMPULSIVE DISORDER

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6.1	Introduction	216
6.2	Neurobiology	216
6.2.1	Brain Imaging Studies of OCD	217
6.2.1.1	Structural Studies	217
6.2.1.2	Functional Imaging Studies	219
6.2.1.3	Magnetic Resonance Spectroscopy	220
6.2.1.4	Neuropharmacological Implications of Brain Imaging Studies in OCD	220
6.2.2	Genetic Studies	222
6.2.2.1	Overview	222
6.2.2.2	Serotonin	222
6.2.2.3	Dopamine	223
6.2.2.4	Glutamate	223
6.2.2.5	Neurotransmitter Metabolism	223
6.2.2.6	Developmental Genes	223
6.2.3	Animal Models of OCD	224
6.3	Clinical Psychopharmacology	225
6.3.1	Effective Monotherapies: Controlled Trials	225
6.3.2	Augmenting Agents	225
6.3.3	Miscellaneous Pharmacological Trials of Interest	234
6.3.4	Induction of Obsessive-Compulsive Symptoms	234
6.3.5	Immunomodulatory Treatments	235
6.4	Experimental Nonpharmacological Treatments	235
6.4.1	Neurosurgery	235
6.4.2	Transcranial Magnetic Stimulation	236
6.4.3	Deep Brain Stimulation	236
6.5	Summary	237
	References	238

6.1 INTRODUCTION

Obsessive-compulsive disorder (OCD) is an anxiety disorder characterized by the presence of unwanted, senseless, intrusive, and distressing thoughts, urges, and images (obsessions) and/or repetitive behaviors (compulsions). These are generally recognized as senseless by the patient. The disorder is relatively common, affecting 2–3% of the population [1, 2]. The content of these thoughts and behaviors is often related to fear of danger to self or others; contamination concerns are particularly frequent. At least half of OCD patients have their onset before the age of 15 [3], and there is evidence (reviewed below) that early-onset patients differ somewhat in comorbidity and underlying neurobiology.

The majority (over 70%) of OCD patients have other comorbid disorders [3, 4]. The pattern of comorbidity is of great interest, as early-onset patients differ in that movement disorders such as Tourette's disorder (TD) or chronic motor tics are strongly comorbid, as is attention-deficit hyperactivity disorder (ADHD) [5, 6]. Comorbid patterns are rather nonspecific for adult-onset OCD cases but include both bipolar disorder and schizophrenia. A subgroup of childhood-onset cases is believed to have onset of OCD in relation to infection with group A β -hemolytic streptococcus, presenting a parallel condition to that of Sydenham's chorea [7, 8].

While OCD is classified as an anxiety disorder in the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV), it is given a separate chapter here as the clinical phenomenology, drug treatment response, family studies, and brain imaging data support a separate neurobiological classification for this disorder and a distinct neuropharmacological profile as well. OCD is considered a major cause of disability worldwide [9]; in spite of this, most treatment research is relatively recent with the bulk carried out in the past two decades.

This chapter presents a selective review on the neurobiology, genetics, and neuropharmacology of OCD. For a more general recent clinically oriented review of the diagnosis and treatment of this fascinating syndrome, see Jenike (2004) [10]. As is evident from this chapter, there have been significant advances in the past two decades, but there remain major opportunities for more specific understanding of this complex and probably heterogeneous illness and its treatment.

6.2 NEUROBIOLOGY

Despite the considerable interest and surge of recent work in this area, the biological basis for OCD is unknown. Clinical phenomenology has provided some important clues to the underlying neurobiology of this disorder. For example, there are striking comorbid clinical associations between OCD or OCD-like phenomena and known brain disorders, typically those involving the motor system, which led to the hypothesis that OCD is a basal ganglia disorder. These observations are not new. In his original 1885 description of basal ganglia syndromes, Gilles de la Tourette described children with senseless rituals and tormenting obsessive thoughts associated with the tics and other movements of the syndrome that bears his name. Osler, in his 1894 monograph *On Chorea and Choreiform Affections*, describes cases with chorea and classical obsessions and compulsions [11]. The initial model of OCD [12–14] formulated a frontal-striatal circuit that is dysregulated in OCD. Research on

the motor system involving the planning of complex actions has proved to be particularly pertinent to OCD and most productive for clinical and brain imaging studies over time. The group of childhood-onset cases that appear to have onset of OCD in relation to group A β -hemolytic streptococcus infection provides another example of a Sydenham's chorea-like movement disorder associated with OCD and suggest a possible autoimmune subgroup [7, 15]. In spite of research implicating abnormal activity in cortical-subcortical circuitry, it is still not clear which aspects of these abnormalities are correlates and which are underlying causes of OCD. As the imaging literature has expanded, the proposed circuitry of OCD has become more complex, as discussed more fully below (and see Figs. 6.1 and 6.2 below).

6.2.1 Brain Imaging Studies of OCD

6.2.1.1 Structural Studies. Brain imaging studies have been the most important source of data concerning underlying brain abnormalities in OCD. Initial anatomic neuroimaging studies using computerized tomography (CT) and anatomic brain magnetic resonance imaging (MRI) found increased ventricular volumes in a pediatric sample [16] and smaller caudate volumes in male adolescents with childhood-onset OCD [17]. MRI studies have generally found enlarged ventricular volumes, but basal ganglia volumes have been variously reported as both increased and decreased [18, 19]. Increased thalamic volumes have also been found in drug-naïve patients [20]. The most convincing lesion studies involving OCD are by far the reports of OCD onset in relation to basal ganglia lesions secondary to stroke (e.g., [21]). The variability in these volumetric findings may reflect heterogeneity in the OCD population, possibly due to differences between childhood- and later-onset populations. The latter group has been most consistent with respect to basal ganglia and orbital frontal cortex (OFC) reduction [19, 22].

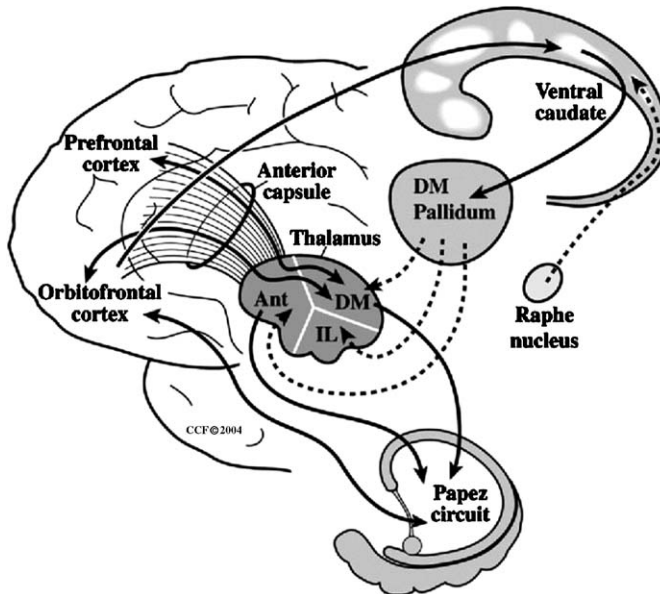


Figure 6.1 Anatomic schematic of OCD neural circuitry. (From [43].)

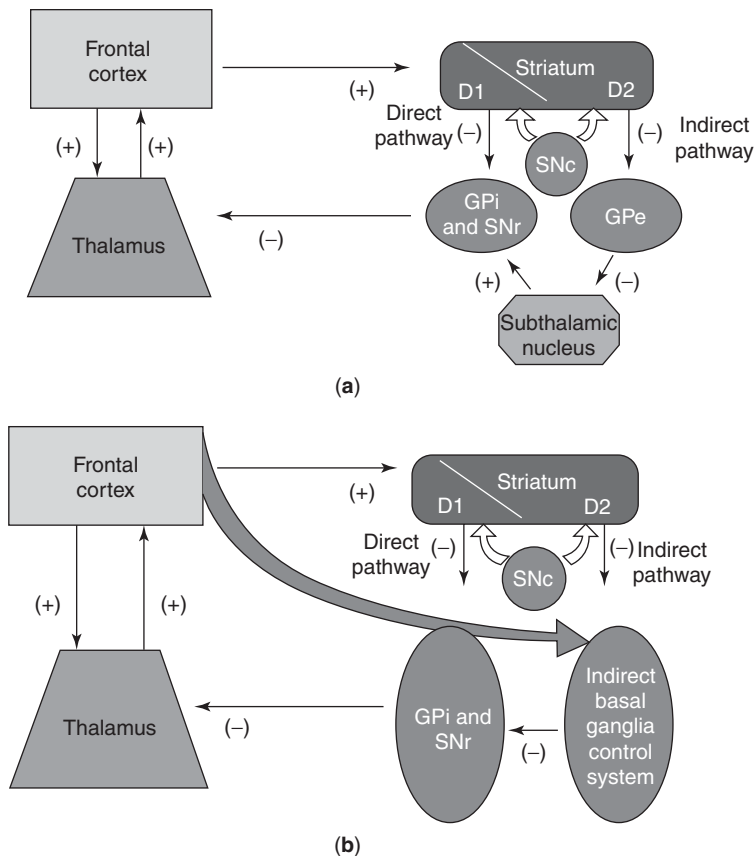


Figure 6.2 (a) Classic conception of direct and indirect frontal-basal ganglia-thalamocortical pathways. GPi and SNr, globus pallidus interna/substantia nigra, pars reticulata complex; GPe, globus pallidus externa. The frontal-subcortical circuit originates in the frontal cortex, which projects to striatum. The direct pathway projects from striatum to the GPi/SNr complex (the main output station of the basal ganglia), which projects to the thalamus, which has reciprocal, excitatory projections to and from the cortical site of origin. This pathway contains two excitatory and two inhibitory projections, making it a net positive-feedback loop. The indirect pathway also originates in the frontal cortex and projects to the striatum but then projects to the GPe, then to the subthalamic nucleus, then back to GPi/SNr, before returning to the thalamus and, finally, back to the frontal cortex. This indirect circuit has three inhibitory connections, making it a net negative-feedback loop. (b) Current conceptualization of prefrontal-basal ganglia-thalamocortical circuitry. Recent anatomic studies have called into question previous views of basal ganglia circuitry. Here, we refer to an indirect basal ganglia control system that consists of the GPe and the subthalamic nucleus. Connections within these structures are more complex than previously thought. The prefrontal cortex has excitatory projections to indirect pathway structures. In addition, the GPe directly projects to the GPi/SNr complex. Nevertheless, the net effect of activity in the indirect circuit still appears to be inhibition of the thalamus, thereby decreasing thalamocortical drive. The frontal-subcortical circuits originating in the lateral prefrontal cortex, orbitofrontal cortex, and anterior cingulate gyrus all pass through subcompartments of the medial dorsal nucleus of the thalamus. (From [18].)

6.2.1.2 Functional Imaging Studies. It is beyond the scope of this chapter to provide a thorough review of functional imaging studies in OCD. These studies have been particularly impressive in the degree of agreement across studies and methods and in their implications for treatment mechanisms. Functional methods have included both positron emission tomography (PET) and single photon emission computed tomography (SPECT) measuring cerebral blood flow (CBF) or metabolism and functional MRI (fMRI) measuring regional blood flow. Several functional studies have compared patients and controls in the resting state; others have scanned patients during symptom provocation or before and after treatment. While no finding is universally replicated, PET studies have generally shown elevated metabolism or regional cerebral blood flow (rCBF) in the OFC, anterior cingulate cortex, and basal ganglia [23]; these increases generally correlate with OCD symptoms.

Neuroimaging studies involving symptom provocation found strong correlations between OCD symptom expression and brain activation in the same regions found to be overactive, namely the OFC, anterior cingulate, and thalamus [24]. A recent SPECT study found greater responsivity of basal ganglia to symptom provocation in responders to sertraline [25]. Specifically, increases were seen in the OFC in the right caudate nucleus, left anterior cingulate (AC), and bilateral OFC. When the study was repeated with fMRI, again the right caudate, bilateral OFC, AC, and right caudate showed increased blood flow. Taken together, these studies link the expression of OCD symptoms with activation of the OFC, basal ganglia, thalamus, and limbic and paralimbic structures predominantly in the right hemisphere. In keeping with these findings, functional neuroimaging studies indicate that OCD patients show a different activation pattern [mesial temporal rather than striatal activation during learning of an implicit (procedural) sequence learning task], suggesting an alternate compensatory pathway for these subjects due presumably to striatal dysfunction. Further, these abnormalities appear specific to OCD [26].

Functional neuroimaging studies of OCD patients before and after treatment generally show decreases in the OFC or in the caudate nuclei in responders to treatment, since reductions in the caudate were seen both with fluoxetine and cognitive behavior therapy (CBT) [27]. Significant decreases were found in bilateral caudate glucose metabolism in responders to CBT compared with nonresponders [28]. These findings were not found in patients with depression, and so these appear to be disease-specific relationships. Another comprehensive examination of neural correlates of response to paroxetine in major depression, OCD, or concurrent OCD and depression also showed some diagnosis-specific differences. Treatment-related decreases in the OFC, thalamus, and ventrolateral prefrontal cortex (VLPFC) were noted for OCD responders; responders with major depression exhibited relative decreases in a large frontal area encompassing the VLPFC and medial, inferior, and dorsolateral frontal cortex. Thus, the drug-induced changes differed with respect to diagnosis and (not shown) paroxetine response versus nonresponse [29]. Perhaps most impressive has been the consistent finding that *elevated* OFC metabolism or blood flow predicts *greater* clinical response to treatment with a serotonin reuptake inhibitor (SRI). This finding has held across several functional imaging modalities and across several different SRIs.

Finally, abnormalities in the serotonin and dopamine transporter availability in unmediated OCD patients [30–33], as shown by SPECT, indicate both serotonergic and dopaminergic dysfunction in patients with OCD (although a PET study found

no difference in serotonin transporter availability in OCD [33]). These important findings are being explored with higher resolution studies. They are of particular interest because of the consistent evidence, reviewed below, that low doses of dopamine antagonists potentiate the effect of selective serotonin reuptake inhibitors (SSRIs), suggesting that overactivity of the dopamine system is part of the pathophysiology of OCD.

6.2.1.3 Magnetic Resonance Spectroscopy. Most magnetic resonance spectroscopy (MRS) studies of OCD have been conducted by Rosenberg and colleagues, who found significantly increased caudate glutamatergic concentration in pediatric OCD patients compared to healthy controls [34] and also found striking decreases in glutamate concentration in the caudate in 11 children with OCD after treatment with paroxetine [35]. These findings are consistent with PET studies of adult patients indicating increased blood flow and metabolism in treatment-naïve OCD patients. However, this drop in glutamate concentration appeared specific to SSRI treatment as it did not hold up for a group of OCD children responding to CBT [36]. It is notable that only caudate and not occipital glutamate concentration decreased, suggesting a localized effect of the drug. These studies, while intriguing, were done without sufficient resolution to distinguish glutamine and glutamate, and thus further understanding of these findings awaits replication with higher field strength machines.

Thus, taken together, and independent of imaging modality or type of treatment used, neuropharmacological hypotheses of OCD pathophysiology have supported the initial models put forward by Rapoport and Wise [37], Modell [12], Insel [37, 38], and Baxter [39] implicating cortical–striatal–thalamic–cortical (CSTC) loops. All of these models build on the discrete, parallel, neuroanatomic circuits connecting the prefrontal cortex, basal ganglia, and thalamus [40], although Saxena and colleagues [18] have summarized the more complex connections now documented within these structures and recent anatomic studies in primates. For example, there is a direct (excitatory) project from prefrontal cortex to the indirect (inhibitory) pathway structures. In light of the unique pattern of relationships between OCD and movement disorders, it is particularly interesting that the cortical–striatal connections provide a common substrate for both thoughts and planned movements. The excitatory projections in these circuits predominantly use glutamate as a neurotransmitter, while inhibitory ones mainly employ γ -aminobutyric acid (GABA). Several peptide transmitters also have roles within these pathways [41]. Other neurotransmitters (serotonin, dopamine, acetylcholine) modify the activity of projections between these structures. The net effect of the current model for OCD, in any case, is of an imbalance between direct and indirect pathways that leads to net overactivity of frontal–subcortical circuits.

6.2.1.4 Neuropharmacological Implications of Brain Imaging Studies in OCD. The imaging studies are almost all in agreement about the importance of the OFC, anterior cingulate, and ventral striatum. As mentioned, most consistent evidence relates OFC activity and treatment response. However, while the studies summarized above provide intriguing information about the pathways mediating OCD thoughts and behaviors, none has addressed the question of whether these findings are correlates of illness or trait markers. In fact, the normalization of caudate metabolism/blood flow with treatment and the normalization of caudate glutamate (Glx)

elevation with drug treatment support imaging results as being largely state rather than trait markers. These results, together with the absence of family studies, make these findings generally ambiguous with respect to the usefulness of imaging measures as intermediate phenotypes for genetic studies.

The major neuropharmacological implications of the model is that there is increased signaling from OFC to subcortical structures (i.e., ventromedial caudate and medial dorsal thalamus). This leaves numerous possible anatomic mediators for this effect, and the imaging data do not support any single pathway. There are strong data indicating that serotonergic systems modulate OCD symptoms, as reviewed in Section 6.3.

Inhibitors of the serotonin (5-HT) transporter [5-HT reuptake inhibitors (5-HT-RIs)] produce at least some clinical benefit in most patients with OCD. Dopamine systems are also suggested by the demonstration that dopamine-blocking agents may potentiate the response to SSRI drugs [42] and, as reviewed below, for some patients this mechanism seems to have particular importance. Other neurotransmitter systems have been implicated on the basis of either the circuitry described above or brain imaging studies and include glutamate, GABA, substance P, and cholinergic and (on the basis of symptom exacerbation by naloxone, an opiate antagonist) endogenous opioid mechanisms.

Perhaps the selective regional (caudate) change in glutamate in blood flow following SSRI treatment provides the most novel treatment-related information. Initial studies in adult OCD showed that the increased metabolic rates associated with OCD symptom severity decreased after SSRI treatment [27]. However, as the major excitatory neurotransmitter system in the circuitry subsuming OCD is glutamatergic and the majority of axon terminals in the caudate nucleus are glutamatergic, stimulating 5-HT, that is, via 5-HT_{2A} receptors, would be expected to decrease glutamatergic efferents from the prefrontal cortex to the caudate nucleus with resulting decreased caudate glutamate concentrations. To date, there have been no studies of glutamatergic agents as monotherapies for OCD that might address this model. Future imaging studies can further extend this model (e.g., through blood flow or metabolic studies of the effects of D₂-blocking augmenting agents).

As seen in Figure 6.1, three general circuits have been assumed based on clinical, imaging, and lesion studies [43]. The first is an orbitofrontal–dorsomedial thalamic loop by way of the internal capsule. This is likely to be glutamatergic [12]. The second involves the frontal cortex–ventral caudate, dorsomedial pallidum and the intralaminar, anterior and dorsomedial thalamic nuclei. While there are multiple neurotransmitters involved in these circuits, including substance P and GABA, there are also serotonergic projections to this component from the dorsal raphe to the ventral striatum. These projections are speculated to be inhibitory. Finally, the third component includes the limbic structures and accounts for the strong anxiety component of OCD; this circuit has strong projections from the anterior cingulate cortex.

Figure 6.2 is an update to our understanding of the circuitry of the basal ganglia–thalamic–frontal loops [18]. It is included because the close association between various movement disorders, basal ganglia lesions, and OCD provides one of the unique and important leads to understanding this illness. Since the original basal ganglia models were postulated, there have been some changes to how the model of basal ganglia circuitry is viewed, most prominently the direct connection between the frontal cortex and the internal pallidum (GPe).

While it is unlikely that one neurotransmitter system will explain the basis for this complex and heterogeneous disorder, efforts have centered largely on the role of the neurotransmitter serotonin. The serotonin hypothesis of OCD that this disorder is in some way due to 5-HT dysfunction, stems from drug treatment studies [44, 45]. As reviewed below, over two decades of work has documented the efficacy and relative (to placebo and to noradrenergic antidepressant drugs) specificity of the anti-OCD effect of 5-HT uptake inhibitors [46]. However, peripheral markers of 5-HT function have not consistently indicated an abnormality in untreated patients with OCD, and exacerbation of OCD by oral administration of the 5-HT agonist m-CPP has not been consistently found [47]. Some evidence for the serotonin hypothesis has been from the genetic studies reviewed below.

Perhaps the most direct relevance of imaging studies for neuropharmacology comes from the rather consistent data on regional change in MRS or functional imaging patterns with treatment. For example, a pattern found in pre- and posttreatment studies with pediatric OCD was consistent paroxetine-induced normalization in regional (i.e., reduction in caudate but not occipital) Glx levels that correlate with treatment response [34].

6.2.2 Genetic Studies

6.2.2.1 Overview. A systematic approach to understanding the genetics of OCD would include twin, family, segregation, linkage, and association studies. Twin studies indicate concordance for OCD symptoms ranging from 26 to 87% with widely varying phenotypic definitions and sample sizes [48]. Family studies support a genetic component for OCD, particularly for populations with early age at onset. The presence of tics in probands may also predict greater familiarity, as summarized by Grados et al. [49]. The findings appear complex, and heterogeneity by phenotype and by gender appears probable [50].

Genetic studies have been based on localization from linkage and from known pharmacological response. It is particularly encouraging that a 9p linkage site has recently been replicated [51, 52]. Because of the selective response of OCD to SRIs, the serotonin system has been the source for putative candidate genes. Cytogenetic studies, the studies of physical duplication, deletion, or disruption of chromosomes, are unexplored in OCD. One exception is the study of the 22q11 deletion, also known as velocardial facial syndrome (VCFS), a 3-MB microdeletion best recognized for its medical complications and association with psychosis [53]. However, OCD has also been associated with this same deletion in several studies, particularly for early-onset OCD [54, 55]. Segregation and linkage studies will not be reviewed here, but we will touch on candidate genes, which were targeted because they were involved in the metabolism of CNS neurotransmitters. Only studies with at least one independent replication are mentioned.

6.2.2.2 Serotonin. The serotonin transporter SLC6A4 on chromosome 17 is a target of SRIs. These drugs increase serotonin in the synapse by decreasing the transporter action. A functional gene 44-bp insertion/deletion polymorphism (*s* and *l* variants) within a gene promoter region (*5-HTTLPR*) has been identified and primarily *l* variants have been implicated [56], but there have been several non-replications. This gene is highly evolutionarily conserved and regulates the entire

serotonergic system. Both the serotonin transporter and some serotonin receptor subtypes such as 5-HT_{2A} and 5-HT_{2C} are highly expressed in the ventral striatum where they could influence the CSTC in OCD [57]. The gene has shown signals for multiple diagnoses, including ADHD, autism, bipolar disorder, and TD. The *ss* genotype is associated with poorer therapeutic response during treatment with antidepressant serotonin transporter (SERT) antagonists, the SSRIs. The 5-HT_{1D} *beta* receptor gene has variants of the coding region which have been studied with preferential transmission of variant G861C (C-to-G) substitution preferentially transmitted to affecteds [58, 59] with other partial replications [60].

6.2.2.3 Dopamine. Both animal and human imaging studies have underlined the involvement of the dopaminergic system in repetitive behaviors [61, 62]. The association with movement disorders and basal ganglia disease and the well-documented efficacy of the D₂-blocking agent haloperidol as an augmenting agent all implicate the dopamine system in OCD. For a recent review, see [63]. There is evidence that the dopamine transporter density in the basal ganglia differs in unmedicated OCD patients in comparison to normal controls [30], suggesting further that the dopaminergic neurotransmitter system is involved in the pathophysiology of OCD. The *DRD4* gene codes for a receptor with several functional polymorphisms in the form of variable numbers of tandem repeats (VNTR) identified within a particular intracellular peptide segment. The 7 repeat has been linked to ADHD, but several studies have found this association with OCD (although the specific allelic associations have been inconsistent [64–67]).

6.2.2.4 Glutamate. Several lines of evidence suggest that OCD could be a consequence of glutamatergic dysfunction. Brain imaging profiles (reviewed above) using a variety of neuroimaging techniques demonstrate alterations in the OFC, basal ganglia, and thalamus which normalize with treatment [68, 69]. These regions are linked in circuits within which glutamate is the primary excitatory neurotransmitter [70–72]. Moreover, a transgenic mouse model (see below) suggests aggravation of TD/OCD-like behavior with glutamatergic drugs [73]. Positive associations have been reported for NMDA receptors (*GRIN2B*) [72] and for *GRIK2* (a kainate receptor) recently also associated with autism [71].

6.2.2.5 Neurotransmitter Metabolism. The X chromosome *MAOA* gene encodes monoamine oxidase A which is found in serotonergic and catecholaminergic brain neurons. (There are, however, no controlled studies indicating efficacy of MAO inhibitor drugs in OCD.) A polymorphism *MAOA* Eco/Rv was found to be significantly more frequent in OCD females in two studies [74, 75]. The enzyme catechol-*O*-methyl transferase (COMT) is the major degradation enzyme for catecholamine neurotransmitters. A Val–Met substitution polymorphism in codon 158 results in low enzymatic activity [76]. COMT's location in the 22q11 region associates it with VCFS, and, as mentioned above, children with VCFS frequently manifest anxiety and OCD [54].

6.2.2.6 Developmental Genes. There have been isolated reports of genes related to neurodevelopment. *Hoxb8*, a member of the mammalian homeobox-containing group of transcription factors, is of interest as disruption shows excessive grooming

in mice [77, 78]. The developmental gene brain-derived neurotrophic factor (BDNF) was found to show significant association with OCD [79]. This is an interesting, if nonspecific, finding which bears replication.

Although genetic studies of OCD have been productive in yielding several replicated significant associations, none yet leads to new insight with respect to the neuropharmacology of OCD. One exception may be the finding of association with the mu opioid receptor [80] (see discussion of new clinical agents in Section 6.3).

6.2.3 Animal Models of OCD

Animal models are of interest in OCD for several reasons. Many OCD habits such as grooming, checking, and avoidance of fecal contamination have led to ethological models for this disorder. This implies the notion of highly conserved neural circuitry for such behavior. Unfortunately, efforts to elucidate the neural circuitry subserving OCD have been hampered by the lack of good animal models and, while some appealing “semiclinical” models (such as canine acral lick) have been published [81], other models have provided contradictory evidence [82–84]. Unlike movement disorders, many of the behaviors that are seen in OCD are inherently unique to humans (e.g., the belief that the thoughts or behaviors are senseless).

One proposed model is schedule-induced polydipsia (SIP). Food-deprived rats drink excessively when exposed to an intermittent feeding schedule, and SIP may be a displacement behavior in response to stress [85]. SSRIs reduce SIP and displacement behaviors after 14–21 days of treatment, suggesting a parallel with treatment of OCD in humans [86]. This parallel may be deceiving, however, as other antianxiety agents may increase SIP [87]. Some animal models are intriguing, such as the quinpirole (an agonist of D₂-like dopamine receptors) rat model in which repetitive cleaning behaviors are partially ameliorated by clomipramine [84]; this model focuses interest on the role of dopamine in the disorder [88]. In spite of appealing face validity (e.g., [89]), a variety of neurotransmitter systems are implicated [90]. A transgenic mouse model of comorbid TD and OCD was created by expressing a neuropotentiating cholera toxin transgene in a subset of dopamine D₁ receptors expressing neurons thought to induce cortical and amygdala glutamate output [91, 92]. It is anticipated that future models using the serotonin transporter and other putative genes will be fruitful.

To date, however, the best animal models have been inspired by the known brain circuitry of OCD. A series of studies from France has addressed regional control within the basal ganglia in primates that mediate some stereotypic and ticlike movements which might be seen as relevant to TD or possibly OCD [93, 94]. These studies utilized microinjections of bicuculline, a GABA antagonist, into various territories of the external globus pallidus (GPe). The regions were selected based on the known striato-pallidal projections to sensorimotor, associative, and limbic regions. The authors hypothesized that while sensorimotor territory injections would induce reversible abnormal movements, injections into the associative and limbic territories of the GPe would produce behavioral disorders. In fact, the bicuculline microinjections induced stereotypy when performed in the limbic part of the GPe (and ADHD-type behaviors when performed in the associative part); this is of particular interest when taken together with the modified view of the prefrontal–basal ganglia circuitry (see Fig. 6.2b) in which direct frontal GPe connectivity is now included in the model.

6.3 CLINICAL PSYCHOPHARMACOLOGY

This section focuses on selective studies addressing the major neuropharmacological issues in OCD, but excellent more general treatment reviews are available [95].

6.3.1 Effective Monotherapies: Controlled Trials

The past two decades have seen highly consistent findings that drugs that inhibit reuptake of serotonin either nonselectively (clomipramine) or selectively provide significant benefit for OCD. The major controlled trials are summarized in Table 6.1. Where pediatric trials have also been carried out, these are included. The selective effect of the SSRIs is particularly important here, as one or two double-blind comparisons with selective noradrenergic uptake inhibitors (e.g., desipramine [96]) show no efficacy of the noradrenergic medication. The well-documented efficacy of SSRIs for OCD has transformed the treatment of the disorder which earlier was treated predominantly with psychotherapy. It further differentiates OCD from depression and other anxiety disorders which may respond well to other agents predominantly affecting NE metabolism.

Table 6.1 contains representative high-quality studies documenting the efficacy for the serotonin uptake inhibitors in OCD. While clomipramine had a slightly more troublesome side-effect profile (including dry mouth, constipation, and some EKG changes), there is some consensus that it is more effective than the more selective serotonin uptake inhibiting agents. It is beyond the scope of this chapter to review the adverse effects of each of the drugs presented here. While the SSRIs are the best tolerated medications for OCD, some subjects, particularly children, have significant side effects, such as agitation, nausea, and in a small percent of cases an increased risk of suicidal ideation [115, 116]. However, generally the effect size for SRIs in OCD is substantial, and the suicidal thoughts occur early in treatment and can be handled with careful monitoring during this period.

It is also beyond the scope of this chapter to review behavioral treatment of OCD, but the reader should be aware that many find cognitive behavior therapy to be as important or more so than drug treatment for this condition [117, 118].

6.3.2 Augmenting Agents

Because the majority of patients classified as “responders” have only partial response to monotherapy with serotonin drugs, it is significant that a small but important group of controlled trials has shown benefit from the addition of another agent. These are considered augmenting agents as their effect appears to be only in conjunction with a SRI. Benefit has been shown most consistently for typical and atypical antipsychotic agents. Pharmacological augmentation strategies for treatment-resistant OCD are also well covered in a recent review [119]. Table 6.2 shows controlled trials for augmenting agents in OCD.

Table 6.2 contains a selective list of high-quality placebo-controlled studies that document the efficacy of typical and atypical antipsychotics as augmenting agents added to selective or nonselective SRIs. A single, as yet unpublished controlled trial also documented the efficacy of clonazepam as an augmenting agent [128], but the typical and atypical antipsychotics remain the only group of medications for which

TABLE 6.1 Serotonin Uptake Inhibitor Efficacy in OCD

Study	N	Design and Dose	Results	Comments
Clomipramine				
Adults: Katz et al., 1990 [97]	263	10 wks; 100–300 mg/d	> 50% of CMI group (vs. <5% for placebo) became subclinical, based on NIMH-OC ($p < 0.001$)	Efficacy shown in acute trial and maintained in a 1-yr DB extension
Children: DeVaugh-Geiss et al., 1992 [98]	60	8 wks; max. daily dose: 3 mg/kg or 200 mg, whichever less	Mean CY-BOCS reduction: 37% (vs. 8% for placebo) ($p < 0.05$)	Side effects typical of tricyclic antidepressants; efficacy maintained in 1-yr OL extension
Fluoxetine				
Adults: Montgomery et al., 1993 [99]	214	8 wks; fixed dose: 20, 40, 60 mg/d	40 and 60 mg were superior; response rates (Y-BOCS and CGI): 40 mg: 48%; 60 mg: 47% (vs. placebo: 26%, 20 mg: 36%) ($p < 0.05$)	16-wk controlled extension offered for responders; response maintained; nonresponders improved in OL extension on 60 mg
Adults: Tollefson et al., 1994 [100]; Tollefson et al., 1994 [101]	355	13 wks; fixed dose: 20, 40, 60 mg/d	All doses superior to placebo; response rates (Y-BOCS): 32–35% for all doses (vs. 8% for placebo) ($p < 0.001$)	13 wks was minimum to see effects of 20 mg; in OL extension, responders maintained acute trial gains and 60 mg yielded added benefit; late-emergent adverse events: asthenia (11%), rhinitis (10%), flu syndrome (10%), abnormal dreams (9%)
Children: Geller et al., 2001 [102]	103	13 wks; max. dose: 60 mg/d	Response rates (CY-BOCS): 49% (vs. 25% for placebo) ($p = 0.03$)	20–60 mg/d effective and well tolerated; side effects and drop-out rates similar for drug and placebo
Fluvoxamine				
Adults: Goodman et al., 1997 [103]	320	10 wks; max. dose: 300 mg/d	Response rates (CGI): 43% (vs. 11% for placebo) (p not provided)	Of all on fluvoxamine, mean Y-BOCS at wk 10 still in moderate range; of responders, mean decreased to mild range
Adults: Hollander et al., 2003 [104]	253	12 wks; 100–300 mg/d of CR fluvoxamine	Response rates (CGI-I): 44% (vs. 23% for placebo) ($p = 0.002$)	Excluded from study if history of nonresponse to SRIs; response seen at wk 2; CR allows for more aggressive dosing

Children: Riddle et al., 2001 [105]	120	10 wks; 50–200 mg/d	Based on CY-BOCS, significant differences seen as early as wk 1 ($p = 0.007$); response rates (CY-BOCS): 42% (vs. 26% for placebo) ($p = 0.06$)	Rapid onset of action
Sertraline				
Adults: Greist et al., 1995 [106]; Greist et al., 1995 [107]	324	12 wks; fixed dose: 50, 100, 200 mg/d	Pooled sertraline group better than placebo, based on Y-BOCS ($p = 0.006$) and CGI-I ($p = 0.01$); response rates (CGI-I): 39% (vs. 30% for placebo)	Larger placebo effect than usual; those completing 3 mos of sertraline continued to improve during 40-wk extension
Adults: Koran et al., 2002 [108]	223	649 pts in 16-wk, flexible dose, SB trial; responders got additional 36 wks SB; 223 responders at 52 wks got 28 wks of 50–200 mg/d DB	During DB, sertraline better than placebo on 2 of 3 primary outcomes: dropout due to relapse/poor response (9% vs. 24%) ($p = 0.006$) and acute exacerbation of symptoms (12% vs. 35%) ($p = 0.001$)	Long-term treatment generally well tolerated; efficacy sustained among prior responders
Children: March et al., 1998 [109]	187	12 wks; up to 200 mg/d at 4 wks, then maintained for 8 more wks	Response rates (CGI-I): 42% (vs. 26% for placebo) ($p = 0.02$)	Short-term safety and effectiveness shown; efficacy differences seen at wk 3 and persisted
Paroxetine				
Adults: Hollander et al., 2003 [110]	348	12 wks; fixed dose: 20, 40, 60 mg/d	40 and 60 mg/d effective ($p < 0.05$); mean CY-BOCS reduction: 40 mg: 25%, 60 mg: 29% (vs. placebo: 13%, 20 mg: 16%)	Long-term effectiveness shown (263 acute trial responders enrolled in 6-mo, flexible dose, OL trial; 105 responders to that OL trial randomized to 6 mo, fixed dose, DB trial); some mild discontinuation symptoms if abruptly stop

(continued)

TABLE 6.1 (Continued)

Study	N	Design and Dose	Results	Comments
Adults: Kamijima et al., 2004 [111]	191	12 wks; 20–50 mg/d	Response rates (Y-BOCS): 50% (vs. 24% for placebo) ($p = 0.0003$)	Effective and generally well tolerated; those not adequately responding to suggested dose of 40 mg/d may benefit from 50 mg/d
Adults: Zohar & Judge, 1996 [112]	406	12 wks; paroxetine: 20–60 mg/d; CMI: 50–250 mg/d	Response rates (Y-BOCS): 55% for paroxetine ($p = 0.001$) and 55% for CMI ($p = 0.005$) (vs. 35% for placebo)	Paroxetine more effective than placebo and comparable to CMI; paroxetine better tolerated than CMI, based on some measures; more anticholinergic adverse events for CMI (53%) than paroxetine (28%), 12% for placebo
Children: Geller et al., 2004 [113]	207	10 wks; 10–50 mg/d	Response rates (CY-BOCS): 65% (vs. 41% for placebo) ($p = 0.002$)	Effective and generally well tolerated; especially gradual titration and tapering suggested due to nonlinear pharmacokinetics
Citalopram				
Adults: Montgomery et al., 2001 [114]	401	12 wks; fixed dose: 20, 40, 60 mg/d	Response rates (Y-BOCS): 20 mg: 57%, 40 mg: 52%, 60 mg: 65% (vs. 37% for placebo) ($p < 0.05$)	All three doses more effective than placebo; need larger sample to see effects of different doses; suggest starting at 20 mg, although 60 mg may be useful if need rapid response

Note: Unless noted, studies are multicenter, randomized, double-blind, placebo-controlled, acute trials with parallel design. Information on extension trials, if conducted, is provided under Comments. CGI: Clinical Global Impressions; CGI-I: Clinical Global Impressions-Improvement scores; CMI: clomipramine; DB: double blind; SB: single blind; NIMH-OC: National Institute of Mental Health Global Obsessive-Compulsive Rating Scale; OL: open label; Y-BOCS: Yale-Brown Obsessive-Compulsive Scale; CY-BOCS: Children's Yale-Brown Obsessive-Compulsive Scale; CR: controlled release.

TABLE 6.2 Controlled Trials of Augmenting Agents in Adults with OCD: Typical and Atypical Antipsychotics

Study	N	Design and Dose	Results	Comments
Haloperidol				
McDougle et al., 1994 [120]	34	OCD pts (with or without a secondary chronic tic d/o) refractory to 8 wks of fluvoxamine (max of 300 mg/d given for over 7 wks) given 4 wks haloperidol or placebo addition; haloperidol dose: 2 mg/d for 3 days, increased by 2 mg every 3 days, to a max of 10 mg/d	Based on Y-BOCS and CGI, 11/17 (65%) responded (vs. 0/17 for placebo) ($p < 0.0002$); of those with comorbid chronic tics, 8/8 responded [vs. 3 (33%) of 9 without tics or family history of tics] ($p = 0.007$)	Support for role of dopamine dysregulation in some OCD patients, particularly those with tic disorder; little or no benefit if no tic disorder; risk of tardive dyskinesia
Risperidone				
McDougle et al., 2000 [42]	36	Patients with primary OCD treated for 6 wks with risperidone ($N = 20$) or placebo ($N = 16$) addition to their SRI; risperidone started at 1 mg/d for 1 wk, with dosage increased 1 mg every week until max of 6 mg/d, as tolerated	Of study completers and based on CGI, 9/18 (50%) responded (vs. 0/15 for placebo) ($p < 0.005$)	Patients with or without comorbid tic disorder or schizotypal personality disorder may benefit; mild transient sedation; risk of tardive dyskinesia
Hollander et al., 2003 [121]	16	Patients treated for 8 wks with risperidone or placebo addition; risperidone initiated at 0.5 mg/d and gradually increased by 0.5 mg/d every 7 days over first 6 wks until max of 3 mg/d or experienced therapeutic effects or side effects	Based on Y-BOCS and CGI, 4/10 (40%) responded (vs. 0/6 for placebo) ($p = 0.12$)	Small sample but consistent with literature indicating 30–50% benefit from atypical antipsychotic addition

(continued)

TABLE 6.2 (Continued)

Study	N	Design and Dose	Results	Comments
Erzegovesi et al., 2005 [122]	39	After 12 wks of OL fluvoxamine monotherapy (max of 300 mg/d; initial study $N = 45$), both responders and nonresponders (total $N = 39$) randomly assigned to 6-wk DB addition of low-dose (0.5 mg) risperidone or placebo; fluvoxamine dose maintained	Significant interaction of “add-on treatment” \times “response” \times “time” ($p = 0.001$); based on Y-BOCS and CGI, during DB phase, 5 (50%) on risperidone and 2 (20%) on placebo became responders	Low dose effective; add-on treatment effective <i>only</i> in fluvoxamine-resistant subgroup; fluvoxamine-responder group had poorer effect of addition than did placebo group
Olanzapine				
Bystritsky et al., 2004 [123]	26	Treatment-refractory patients without significant comorbidity treated for 6 wks with olanzapine (started at 2.5 mg, increased to 5 mg/d for days 4–7, then 5–20 mg/day) or placebo, while continuing their SRI at stable dose	Based on Y-BOCS, 6/13 (46%) responded (vs. 0/13 for placebo) ($p = 0.01$), with mean Y-BOCS decreasing 16% in olanzapine addition group	Effect on OCD symptoms appears not to be due to antipsychotic or mood-stabilizing effects; no extrapyramidal side effects; of olanzapine patients, two (15%) discontinued: one due to lack of effect and sedation, one due to weight gain
Shapira et al., 2004 [124]	44	Partial or nonresponders to 8-wk OL fluoxetine trial (40 mg in almost all pts) treated with 6 wks olanzapine (5–10 mg) or placebo addition to fluoxetine	Groups similar; based on Y-BOCS, both groups improved over time	May have needed longer prior monotherapy

Quetiapine

Denys et al., 2004 [125]	40	Patients without significant comorbidity treated for 8 wks of up to 300 mg/d quetiapine or placebo, in addition to their SRI; quetiapine initiated at dose of 50 mg/d and increased at fixed schedule (wks 1–2: 100 mg/d; wks 3–6: 200 mg/d; wks 7–8: 300 mg/d)	Based on Y-BOCS and CGI, 8/20 (40%) responded [vs. 2/20 (10%) for placebo] ($p = 0.03$)	Effective, especially for severe obsessions; response seen within 4–6 wks; most common side effects: somnolence, dry mouth, weight gain, dizziness
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Buspirone (Anxiolytic)

Grady et al., 1993 [126]	14	Crossover design, 4 wks each, placebo and buspirone addition to ongoing fluoxetine; buspirone dosage increased over 2 wks; all had stable dose of 60 mg/d for final 2 wks of active treatment	No significant effect of drug; only one patient had clinically meaningful response to buspirone (vs. 0 for placebo)	Parallels earlier study by same group showing lack of improvement for buspirone added to CMI; one patient withdrew after a seizure
McDougle et al., 1993 [127]	33	OCD patients refractory to 8 wks of fluvoxamine (up to 300 mg/d) given 6 wks buspirone ($N = 19$; initially 15 mg/d in 3 divided doses, then increased by 15 mg every other day to a max of 60 mg/d in 3 divided doses) or placebo ($N = 14$) addition to fluvoxamine continued at the same dose	No differences: based on Y-BOCS and CGI, 2/19 (11%) responded [vs. 2/14 (14%) for placebo]	Negative study

(continued)

TABLE 6.2 (Continued)

Study	N	Design and Dose	Results	Comments
Clonazepam (Anxiolytic)				
Pigott et al., 1992 [128]	18	2–6 mg/d added to ongoing CMI or fluoxetine (other information not available)	Added benefit demonstrated on 1 of 3 OCD rating scales	Benzodiazepine with preferential effects on serotonergic system; especially helpful if anxiety prominent; improvement seen in hours to days; typically started at 0.5 mg once or twice daily, with max of 5 mg/d when used as an antiobsessional
Desipramine (TCA)				
Barr et al., 1997 [129]	33	Desipramine or placebo added for 6 or 10 wks to SSRI treatment; desipramine daily dose adjusted weekly to obtain plasma level > 125 ng/mL if tolerated; mean final plasma level was 148.3 ng/mL (SD = 82.0)	Based on Y-BOCS, there was no drug-by-time interaction at wk 6 ($p = 0.07$) or wk 10 ($p = 0.45$)	Desipramine's relatively specific inhibition of norepinephrine reuptake was not an effective addition [30 patients completed 6 wks; 25 of these enrolled in extension; 23 (10 on desipramine, 13 on placebo) completed 10 wks]
Nortriptyline (TCA)				
Noorbala et al., 1998 [130]	30	DB nortriptyline (50 mg/d) or placebo added to 150 mg/d CMI for 8 wks	Based on Y-BOCS, both improved; but active addition showed advantage by wk 4 ($p = 0.007$); by wk 8, $p < 0.0001$	Rapid onset of action; (sample mostly female)

Lithium

McDougle et al., 1991 [131]	30	Two trials: 2-wk ($N=20$) and 4-wk ($N=10$) augmentation of ongoing fluvoxamine (200–300 mg/d); lithium initially given at 900 mg/d in 3 divided doses; dosages then adjusted to keep serum levels between 0.5 and 1.2 mmol/L	Significant change in Y-BOCS and CGI in trial 1 ($p<0.05$) but not in trial 2; based on Y-BOCS and CGI, 2/11 (18%) and 0/5 of patients having 2 wks or 4 wks, respectively, of lithium addition were responders (vs. 0/9 and 0/5 for placebo)	Generally not effective; some OL treatment also given subsequently; even 4 wks was not effective for most
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Pindolol

Dannon et al., 2000 [132]	16	6 wks of pindolol ($N=8$; 2.5 mg t.i.d.) vs. placebo ($N=6$) addition to paroxetine (up to 60 mg/d)	Based on Y-BOCS, pindolol was superior to placebo ($p<0.01$) after wk 4	Results based on $N=14$ as two placebo patients dropped out
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Inositol

Fux et al., 1999 [133]	10	Crossover design with 18 g/d inositol or placebo for 6 wks each, in addition to ongoing SRI (dosage range was 40–60 mg/d for fluoxetine; 200–250 mg/d for fluvoxamine; 150–225 mg/d for CMI)	Both groups improved ($p<0.000$); no significant group differences	No added effects from inositol (putative site of action intracellular; SRIs synaptic)
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Note: Unless noted, studies are double blind and placebo controlled with SRI-refractory OCD patients. CGI: Clinical Global Impressions; DB: double blind; OL: open label; SRI: serotonin reuptake inhibitor; SSRI: selective serotonin reuptake inhibitor; TCA: tricyclic antidepressant; Y-BOCS: Yale-Brown Obsessive-Compulsive Scale.

there are really well-documented augmenting effects for the treatment of OCD. Increased clarity regarding criteria for defining treatment resistance and nonresponse would be useful in studies of augmenting agents and treatment in general [134].

6.3.3 Miscellaneous Pharmacological Trials of Interest

Tables 6.1 and 6.2 do not include unblinded trials or double-blind comparisons of active agents that do not also include a placebo. However, there is an intriguing list of agents for which either convincing case series or small controlled trials suggest other pharmacotherapies. Some small open studies indicate that tramadol (a mu opioid agonist) may be efficacious for OCD [135, 136]. Also, anecdotal evidence and a recent small short-term placebo-controlled, double-blind study [137, 138] suggest that oral morphine sulfate may be of benefit in treatment-resistant OCD, although, among other questions, it is unclear how often the drug should be administered. While clonazepam is widely used as an anxiolytic in OCD, a recent double-blind placebo-controlled study showed no evidence for its usefulness as monotherapy [139]. One early study examined clonazepam as an augmenting agent (see Table 6.2); an early crossover study provided initial support for its usefulness as monotherapy [140]). Other studies suggest that venlafaxine, a serotonin–norepinephrine reuptake inhibitor might be effective as monotherapy or augmenting agent [119, 141, 142]; see [143] for a review. A small study of nicotine patches given to nonsmoking OCD patients found significant improvement compared to placebo [144]; this most probably reflects a nonspecific anxiolytic effect of nicotine. Lastly, in light of evidence of hyperactivity of glutamatergic circuits in OCD, an interesting new direction involves riluzole, an antiglutamatergic agent that currently is under open-label study in adults (also, for a related published case report, see [145]).

6.3.4 Induction of Obsessive-Compulsive Symptoms

Evidence is particularly impressive that antipsychotics may augment SRI treatment of OCD. In contrast to this effect is the growing number of case reports documenting the provocation or worsening of OCD with both typical and atypical antipsychotics [146]. There has been some impression that this effect is more salient with clozapine. Speculatively, the 5-HT₂ antagonist effect of the atypical antipsychotics may disinhibit dopamine neurons, producing increased output from the orbitofrontal/cingulate cortex and thus precipitating or exacerbating OCD based on the model circuitry shown above [147]. It also has been speculated that the D₂ and 5-HT₂ receptor occupancy of atypical antipsychotics at low doses may actually antagonize the SSRI effects. Management suggestions have therefore included a trial of increased dose of the atypical agent before discontinuation [148]. Following an analysis of the effects of the nonselective 5-HT receptor agonist mCPP, which worsened OCD symptoms, a small double-blind study found that sumatriptan, a 5-HT_{1D} receptor agonist, also worsened OCD symptoms [149]. Finally, a preliminary study indicated that the mu opioid antagonist naloxone exacerbates OCD symptoms [150], although, paradoxically, another mu opioid antagonist, naltrexone, showed some efficacy in treating compulsive self-injurious behavior [151].

6.3.5 Immunomodulatory Treatments

Based on the observation that some childhood-onset OCD patients had onset apparently in relationship to infection with group A β -hemolytic streptococcus (GABHS), a subgroup of children were proposed who were hypothesized to have an autoimmune-based form of the disorder. As a partial test of this model, two treatments were examined. In the first, a controlled treatment trial of intravenous immunoglobulin (IVIG; 1 g/kg/day on two consecutive days) was carried out. The second was an open trial of plasmapheresis consisting of four to five single volume exchanges over a two-week period. These extremely interesting findings have not yet been replicated. A single trial of penicillin prophylaxis was negative [152]. In spite of growing evidence linking GABHS infection and onset of OCD [153], there remains only a single controlled trial documenting the efficacy of immunomodulatory treatment [154, 155].

6.4 EXPERIMENTAL NONPHARMACOLOGICAL TREATMENTS

In spite of the major advances in drug treatment of OCD over the past two decades, at least 10% of the OCD population remains severely affected. This amounts to hundreds of thousands of patients with a debilitating disease without effective treatment. Recent developments in techniques for direct brain manipulation have been applied both to neurological and psychiatric disorders. Given the close connection between OCD and a variety of movement disorders, it is of great interest that selected neurosurgical lesions such as subcaudate tractotomy and deep brain stimulation (DBS) have been effective in the treatment of Parkinson's and other movement disorders. Selective reviews are given below with special focus on the implications for the circuitry and potential neuropharmacology of OCD.

6.4.1 Neurosurgery

The data on neurosurgical lesion treatment for OCD consist of open trials of small numbers of patients with intractable OCD and typically high rates of comorbid Axis I disorders. Response rates across centers vary from about 20 to 60% with varying diagnostic methods and surgical procedures across centers. No randomized double-blind trials have been reported [156]. Various lesions interrupting the CSTC loops have included: anterior capsulotomy, subcaudate tractotomy, limbic leucotomy, and anterior cingulotomy. These are summarized in Table 6.3 and, as seen, interrupt the major pathways in circuits shown in Figure 6.1.

The focus of neurosurgical lesions for OCD is principally to interrupt frontal–basal ganglia and frontal–limbic connections. Reports of success in OCD patients have followed open trials of anterior capsulotomy, a lesion that aims to target connections between dorsomedial thalamus and orbital and medial prefrontal cortex. The newer technique of gamma knife capsulotomy has, at least in theory, enabled controlled trials because of the noninvasive nature of a sham lesion. (The sites of neurosurgical lesions are also the focus for trials of DBS, which, similarly, lends itself more easily to controlled trials.) It is beyond the scope of this chapter to review these studies in greater detail in the absence of any controlled trials documenting efficacy.

However, it is important to note that the circuitry involved is the same as that implicated by brain imaging studies (see above).

6.4.2 Transcranial Magnetic Stimulation

Transcranial magnetic stimulation [TMS, or repetitive transcranial magnetic stimulation (rTMS)] has been proposed as therapeutic for various psychiatric disorders, mainly depression, although stimulation characteristics remain in dispute [158]. In OCD, TMS studies have been used to explore abnormal responsivity of brain circuits, with preliminary findings of decreased neuronal inhibition and a reduced cortical silent period in the primary motor area for TD and OCD [159, 160]. While preliminary, these studies provide suggestive complementary evidence of abnormalities in cortical motor circuitry. However, to date, TMS does not appear to be therapeutically useful in OCD, although this view is based on small sample studies [161, 162] and only a single controlled trial [161].

6.4.3 Deep Brain Stimulation

The realization that chronic high-frequency stimulation resulted in clinical benefits analogous to those of neurosurgical lesioning transformed the use of functional neurosurgery for the treatment of movement disorders (primarily tremor and Parkinson's disease). The use of the more flexible and reversible DBS techniques, instead of irreversible surgical ablations and tract lesions, has been expanded to the treatment of chronic pain and psychiatric disorders, particularly intractable depression and OCD [163]. The site of stimulation overlaps somewhat with the sites of surgical lesions described in Table 6.3 and Figures 6.1 and 6.2, although there is an impressive case report of ventral caudate stimulation [164]. Thalamic and basal ganglia stimulation are most frequently used for movement disorders [165]. DBS has largely replaced pallidotomy in the treatment of Parkinson's disease.

The clinical applications have preceded the scientific understanding of the mechanisms of action of DBS, and it has been puzzling how stimulation initially thought to activate neurons could have therapeutic outcomes similar to those from lesioning of target structures. Basic work is still going on to address such fundamental topics as the volume of tissue influenced by DBS, the effects on neurotransmitter systems, and functional imaging of DBS, but several alternate hypotheses on the physiological changes induced by stimulation remain. From a neuropharmacological standpoint, it would appear that DBS affects glutamate, dopamine, and GABA systems [165]. To date, only a small number of studies have been published on DBS for OCD. Nuttin et al. [166] reported on the effects of stimulation of the internal capsule of four patients with OCD at a site described as identical to that used in capsulotomy. Beneficial effects were described in three of the four patients. The same group reported on long-term follow-up in six patients [167]. This latter study was of special interest as it included double-blind evaluation with and without the stimulation on. Three of the six were considered responders to chronic stimulation. There also has been a report of a single case with dramatic improvement [168]. Most recent is a study [169] of four cases with a short-term, blinded, off-on design and long-term, open follow-up; one patient improved dramatically during blinded and open treatment and a second showed moderate benefit during open follow-up. DBS

TABLE 6.3 Summary of Procedures for Modern Neurosurgery for Psychiatric Disorders

Procedure	Target	Rationale	Current Indications
Anterior cingulotomy	Anterior cingulum	Disconnect Papez circuit	Affective disorders, OCD, anxiety disorders
Subcaudate tractotomy	Frontobasal white matter	Disconnect frontolimbic connections	Affective disorders, OCD, anxiety disorders
Limbic leukotomy	Anterior cingulum and frontobasal white matter	Cingulotomy + subcaudate tractotomy	OCD, affective disorders
Capsulotomy	Anterior limb of internal capsule	Disconnect frontolimbic and caudate-putaminal connections	OCD, anxiety disorders, panic disorder

Source: From [157].

continues to be of interest as a probe of putative circuitry, as reports of improvement of obsessive-compulsive symptoms in patients with Parkinson's disorder, for example, suggest that subthalamic nucleus stimulation may also prove helpful [170]. For the moment, however, most work involves the placement of probes in the same area where subcaudate tractotomy is carried out, with some electrodes in part of the ventral striatum. It is clear that this is a very early stage of treatment development, but these small careful trials will be of importance in elucidating the circuitry of OCD with possible discovery of numerous possible etiologic sites for the disorder within the same circuitry.

6.5 SUMMARY

The advances of the last two decades have revolutionized our understanding and treatment of OCD. The disorder is now readily diagnosed by health care professionals, and appropriate treatments—drug treatment and behavior therapy—are available. Remarkably, the brain imaging studies have served to differentiate OCD from other anxiety disorders, and there is surprising agreement on the functional and some aspects of the anatomic abnormalities. Less successful have been the candidate gene studies, although the agreement of two independent linkage studies on a 9p locus is important. Also surprising is the degree to which there is some agreement on the gene for the D₄ receptor and 5-HT transporter in OCD. Efforts to subgroup the phenotype in terms of symptom profile have not been particularly successful, and considerably more work needs to be done, perhaps using other more neurobiological measures as alternate endophenotypes. Before this can be done, however, the conflicting data about whether brain imaging results reflect state or trait markers need to be resolved. To this end, twin and family imaging studies are needed for these and other biological measures. Such studies are ongoing at several sites.

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7

PHENOMENOLOGY AND CLINICAL SCIENCE OF SCHIZOPHRENIA

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7.1	Introduction	252
7.2	Clinical Phenomenology and Treatment	252
7.2.1	Psychosis	253
7.2.1.1	Phenomenology	253
7.2.1.2	Treatment	254
7.2.2	Cognitive Dysfunction	254
7.2.2.1	Phenomenology	254
7.2.2.2	Treatment	254
7.2.3	Negative Affect	255
7.2.3.1	Phenomenology	255
7.2.3.2	Treatment	255
7.3	Biological Mechanisms	256
7.3.1	Human Brain Imaging	256
7.3.1.1	Magnetic Resonance Imaging	256
7.3.1.2	Magnetic Resonance Spectroscopic Imaging	256
7.3.1.3	Functional Brain Imaging: fMRI and PET	257
7.3.2	Human Post Mortem	260
7.3.2.1	Structural	260
7.3.2.2	Neurochemical	260
7.3.3	Genetics and Phenotypes	262
7.4	Animal Models	263
7.4.1	Cognitive Deficit Model	263
7.4.1.1	Working Memory Deficits	263
7.4.1.2	Declarative Memory Deficits	263
7.4.1.3	Attention Deficits	263
7.4.2	Genetic Models	264
7.4.3	Psychosis Model	264
7.4.4	Neurodevelopmental Model	265
7.4.4.1	Disruption of Neurogenesis	265
7.4.4.2	Lesion Models	265
7.5	Conclusion: How Far Have We Come and What are the Remaining Questions?	265
	References	266

7.1 INTRODUCTION

Schizophrenia is a chronic psychotic illness with an unknown pathophysiology and etiology. It is the prototypical psychotic illness because of its pervasive and chronic manifestations and its human impact. Because the symptoms have their onset in early adult years and frequently run unabated throughout life, its medical need is high. Treatments are palliative, and partially effective.

The causes and mechanisms of illness will undoubtedly be complex. Neither a single brain region nor a single neurochemical alteration seems likely but several. Brain systems are thought to underlie the symptoms of the illness; some evidence suggests that the limbic system is the substrate for psychotic symptoms, the prefrontal neocortex for cognitive symptoms, and the frontoparietal cortex for the affective symptoms. A genetic vulnerability with environmental determinants of etiology is the theoretical construct guiding research.

Research into the biological basis of schizophrenia has become more informative as the tools and understanding in neuroscience itself have become more sophisticated. It has only been recent that tools for human brain research have been sensitive and reliable enough to contribute. But now, human brain imaging techniques, postmortem tissue analysis, and genetic tools are the main sources of data informing schizophrenia biology. Animal models for aspects of the illness are based on growing knowledge about the brain in schizophrenia and its genetic underpinnings. Advances in the understanding of the biology of schizophrenia are necessary to support new drug development and modify the course of illness.

7.2 CLINICAL PHENOMENOLOGY AND TREATMENT

Schizophrenia is characterized by a constellation of symptoms that include psychosis, cognitive deficits, and negative symptoms [1–7]. The course of schizophrenia is life-long. The illness may have a precipitous onset in the late teens and early adult years followed by an episodic course sometimes with satisfactory recovery between episodes. Often other patterns of illness occur with an insidious onset, partial recovery, or a remarkable lack of recovery between episodes [8, 9]. In most affected individuals, a profound deterioration in psychosocial function occurs within the first few years of the illness [10] and then settles at a low, flatter plateau. Surprisingly, symptoms can improve in later life after 50 years of age. The Vermont study found considerable heterogeneity in outcome in later life, including frank late improvers [11, 12]. These data are consistent with several other outcome studies in Europe and the United States which report frequent good outcome in later years for individuals with schizophrenia [8, 9, 13, 14] even though divergent descriptions exist [15]. It is not known if this is due to the later years being less demanding or if the normal aging process is therapeutic in the illness. Nonetheless, the disease course of schizophrenia can be easily distinguished from traditional neurodegenerative disorders where the course is progressively downhill, such as Parkinson's disease or Alzheimer's dementia, and from traditional neurodevelopmental disorders, such as mental retardation, where the course is low and steady from early years.

The prevalence of 1% in the general population, 9% in siblings of an affected individual, 12% in offspring, and 40–50% in identical twins [16] speaks to a genetic

influence, but one that does not inevitably result in schizophrenia. Schizophrenia can also occur without any family history. Several epidemiological factors have been associated with a predisposition to schizophrenia, including prenatal maternal illness during the second trimester, perinatal birth complications, and winter births [17, 18]. Each risk factor confers a modest risk alone with genetics being the strongest risk factor. Environmental factors contribute to risk, such as adolescent use of cannabis in genetically susceptible individuals [19]. Possibly when they occur together, these risks may be multiplicative. Moreover, the risk factors as a group suggest the importance of early life events in the onset of an illness whose florid symptoms appear much later in life.

Several meta-analyses have demonstrated the clustering of symptoms into at least three distinct symptom domains in schizophrenia: (1) *positive symptoms*, including hallucinations, delusions, thought disorder, and paranoia; (2) *cognitive dysfunction*, especially in attention, working memory, and executive function; and (3) *negative symptoms* such as anhedonia, social isolation, and thought poverty [1–7].

When clinical symptoms are related to imaging findings, specific brain areas are found to be differentially involved in symptom manifestations in schizophrenia. Whether these regionally specific changes are a cause or effect of the disorder is not known, but they do suggest the presence of distinct neuroanatomical substrates, possibly distinct cerebral systems, for the different symptom clusters. Accordingly, we will address these clusters as distinct entities—psychosis, cognitive dysfunction, and negative affect.

7.2.1 Psychosis

7.2.1.1 Phenomenology. Psychosis reflects a loss of touch with reality and distortion of mental functions [20]. These include delusions (distortions in thought content), hallucinations (distortion in perception), disorganized speech (distortions in language and thought process), and disorganized behavior (distortion in self-monitoring of behavior). Delusions are among the most common of the schizophrenia symptoms. They are experiences that persons with schizophrenia believe in fervently although they have no basis in fact. Common examples are delusions of persecution, in which they feel that they are being plotted against, spied on, or intentionally victimized. They may also exhibit paranoia. Other delusions may include those such as grandiosity and religiosity. Bizarre delusions are those that are clearly implausible. Hallucinations may occur in any sensory modality with auditory hallucinations being the most common. These are usually perceived as voices distinct from their own thoughts and often occur as two or more voices conversing with one another or voices maintaining a running commentary on the person's thoughts or behaviors. Disorganized thinking is another key symptom and is evaluated on the basis of the individual's speech. Schizophrenics may rapidly switch from one topic to another lacking a logical connection (loose association), making it difficult to follow their train of thought. A single, unimportant word to the listener may become the focus or topic of the next sentence. The subjects, however, believe that they make perfectly good sense. In severe disorganization, the person may be incomprehensible. Disorganized behavior may manifest in any goal-directed behavior leading to difficulty performing routine daily activities such as preparing a meal or taking a shower. The person may appear disheveled, dress peculiarly, or display inappropriate behavior.

7.2.1.2 Treatment. Antipsychotic drugs have vastly improved the lives of those afflicted with schizophrenia, although they still suffer considerable residual symptom burden and life-long psychosocial impairments. The conventional antipsychotics (butyrophenones, phenothiazines, and thioxanthenes) with potent antidopaminergic activity were used successfully to treat psychosis for 50 years, albeit with acute and chronic motor side effects. In 1990, newer drugs were introduced with higher antiserotonergic potency accompanying the dopamine receptor blockade. These drugs were less likely to cause motor side effects and dysphoria but have their own serious side effects. The metabolic syndrome (weight gain, hyperlipidemia, diabetes, hypertension) is a side effect of concern with many of the new antipsychotics. Clozapine remains the only antipsychotic with demonstrably greater antipsychotic efficacy; the mechanism of its better effect is still not known.

7.2.2 Cognitive Dysfunction

7.2.2.1 Phenomenology. The core symptoms of schizophrenia are of a cognitive nature. The particularly prominent cognitive deficits are working memory defects, attentional dysfunction, verbal and visual learning and memory, processing speed, and social learning [21–26]. No cognitive domains are entirely spared, and deficits in performance are highly intercorrelated within persons [27]. However, schizophrenic subjects in many of the studies show a particular profile of deficits that rules out the lack of motivation as a factor in performance. Neuropsychological characteristics of schizophrenia have not served to localize disease pathophysiology. For example, in schizophrenic persons, memory deficits for recurring digit occur that are consistent with temporohippocampal dysfunction [21]. Functions that are ascribed to frontal cortex are abnormal (e.g., verbal fluency, spatial performance, pattern recognition), and long-term memory is affected. Besides, persons with schizophrenia also perform tasks poorly that require sustained attention or vigilance characteristically associated with the anterior cingulate [28]. Deficits in memory possibly involving the hippocampus occur, including explicit memory, verbal memory, and working memory [29, 30]. Deficits in working memory may explain a part of the disorganization and functional deterioration observed in the illness, since the ability to hold information “on-line” is critical for organizing future thoughts and actions in the context of the recent past [31]. These characteristics of cognition in schizophrenia suggest broad cortical dysfunction.

7.2.2.2 Treatment. Existing treatments of schizophrenia have generally been unsuccessful in treating cognitive deficits in schizophrenia. There is controversy over whether second-generation antipsychotics improve cognition more than classical antipsychotics [32–34]. The MATRICS program was developed to identify potential molecular targets to treat cognitive deficits in schizophrenia [35]. Those targets judged to be most promising include a D_1 dopamine agonist [36], an α_7 nicotinic agonist [37], muscarinic agonists [38], serotonin 5-HT_{1A} and 5-HT_{2A} [39] ligands, noradrenergic agonists, [40] and modulators of the glutamate-sensitive *N*-methyl-D-aspartate (NMDA)-gated ionophore [41]. The metabotropic glutamate receptors mGluR 2, 3, and 5 modulate NMDA receptor function and may also provide a means to enhance cognition [42]. These drugs to improve cognition in schizophrenia are proposed as co-treatments and not as alternatives to current antipsychotics. Thus they will be tested in volunteers whose positive symptoms are optimally treated and

stable. For example, some current strategies being tested include atomoxetine, a norepinephrine (NE) reuptake inhibitor that increases norepinephrine and dopamine levels in the frontal cortex, and M_1 muscarinic agonists (*N*-desmethyl clozapine, a derivative of clozapine with M_1 agonist properties).

7.2.3 Negative Affect

7.2.3.1 Phenomenology. In general, negative symptoms reflect a diminution or loss of normal functions [20]. Affective flattening, alogia, and avolition are prominent features of schizophrenia. Affective flattening is common in schizophrenia and is characterized by the person's face appearing immobile and unresponsive. The range of emotional expressiveness is diminished. Alogia, the poverty of speech, is manifested by short, empty replies. This is due not to unwillingness to speak but rather to diminution of thoughts resulting in decreased fluency and productivity of speech. Avolition is characterized by an inability to initiate and persist in goal-directed activities. The person may sit for long periods of time without showing any interest in participating in work or social activities. These subjects also have poor eye contact and reduced body language. One caveat to keep in mind is that negative symptomatology may be secondary to other factors. For this reason, it is useful to divide negative symptomatology into primary negative symptoms (or deficit symptoms) and secondary symptoms. Primary negative symptoms are the manifestations of schizophrenia and may be complicated by secondary negative symptoms that may occur because of depression, paranoia, or medication side effects. Certain antipsychotics produce extrapyramidal side effects such as bradykinesia that may mimic affective flattening. Comorbid depression can be distinguished by the presence of other symptoms of depression not found in schizophrenia. Paranoia can cause subjects to stay in a room and not talk to people, which can be mistaken as social isolation.

7.2.3.2 Treatment. Specific treatments are not available for primary negative symptoms. Antipsychotic drugs can diminish negative symptoms—an effect that may be secondary to the reduction of acute psychosis. Some studies suggest that the second-generation drugs are effective for secondary negative symptoms but they have shown no efficacy for the deficit symptoms [43]. In a longitudinal study, clozapine was found to be ineffective in treating deficit symptoms [44]. Initial promise of glutamatergic agents such as D-cycloserine [45, 46] has not been replicated in the latest larger multicenter study [47].

The clustering of symptoms and differential response of each symptom domain to medication are consistent with the concept of specific neural substrates for each cluster. While we have an understanding of the pharmacology of receptors targeted by antipsychotic medications, we do not know the cerebral mechanisms of actions of these drugs. It is probable that modulation of receptors at the cellular level can result in functional changes at the neural network level.

Systems biology concepts [48] are beginning to be applied to schizophrenia. Systems neuroscience, the study of the function of neural circuits, is concerned with the functional organization and processing of information in cellular networks, thereby linking molecular and cellular biology to behaviors such as cognitive, motivational, perceptual, and motor processes. In schizophrenia, specific neural

networks may underlie each of the symptom domains. The degree of dysfunction in neural systems may vary and predominantly affected systems may have greater impact on the clinical presentation. The notion is that there are abnormal networks, not just an abnormal protein, in schizophrenia. This neural system-based approach provides a plausible and scientifically sound framework in which to conceptualize the pathophysiology of schizophrenia. We expand on this concept in the next section.

7.3 BIOLOGICAL MECHANISMS

7.3.1 Human Brain Imaging

Advances made in neuroimaging modalities allow regional *in vivo* observations of brain structure, chemistry, and function in persons with schizophrenia.

7.3.1.1 Magnetic Resonance Imaging. Magnetic resonance imaging (MRI) provides structural images of the brain with excellent cross-sectional anatomical detail and strong grey/white matter contrast. MRI studies report a reduction in overall brain size, an increase in ventricular size, and variable cortical wasting in schizophrenia [49–52]. These reports confirm and extend older literature using the computerized axial tomography (CAT) examination of schizophrenia that demonstrated ventricular enlargement [52]. More recently, MRI studies have reported a volume reduction in medial temporal cortical structures (hippocampus, amygdala, and parahippocampal gyrus) [53–57], thalamus [58], and striatum [59, 60]. New analytic techniques for shape analysis show regional shape differences of hippocampus [61] and thalamus [58] in schizophrenia. The volume of the superior temporal gyrus may be reduced in schizophrenia, a change that correlates with the presence of hallucinations [55, 62] and with regional electroencephalographic (EEG) changes [63]. Reduced volume [64–66] and cortical thinning [67] of the cingulate have been described. Frontal lobe abnormalities have been debated with studies showing reduction in volume [68, 69] or no change [70, 71]. The suspicion that a portion of these findings could be caused by antipsychotic treatment was raised by the results of a recent study showing haloperidol-associated increases in ventricular size and decreases in neocortical mass in first-break schizophrenia over the first three months of treatment in a study where second-generation drug treatment produced none of these alterations [72]. The extent to which the overall volume of a brain structure reflects any internal pathology, especially if the pathology is subtle, is necessarily limited. Also, while positive MRI data identify a brain area for further study, negative results do not rule out areas as pathological.

7.3.1.2 Magnetic Resonance Spectroscopic Imaging. Magnetic resonance spectroscopy (MRS) imaging allows measurement of chemical concentrations. Each metabolite is identified by its unique position on a frequency spectrum of multiple peaks; the area under each peak provides a measure of the concentration of the metabolite. Proton MRS (^1H MRS) studies show decreases in *N*-acetyl aspartate (NAA) in the prefrontal cortex [73–75] and temporal lobe [76–80] while others show insignificant trends or no difference [81, 82]. Similarly, reductions of NAA are seen in the anterior cingulate [83] but not in all studies [84]. NAA, found exclusively in

neurons, has been speculated to reflect neuronal integrity. Phosphorous MRS (^{31}P MRS) data reflect the integrity of neuronal cell membranes. Decreased phosphomonoester resonance (precursors of membrane phospholipids) and increased phosphodiester resonances (breakdown products) in the prefrontal and temporal cortices in schizophrenia are postulated to reflect an increased turnover of membranes, possibly due to abnormalities in synaptic pruning [75, 85].

7.3.1.3 Functional Brain Imaging: fMRI and PET. Functional MRI with BOLD (blood-oxygen-level dependent) provides dynamic physiological information and includes the BOLD technique. This technique indirectly measures changes in regional blood flow (rCBF) which reflects regional activity. Positron emission tomography (PET) imaging involves the use of a radioactive tracer to measure rCBF metabolic activity, using O-15 water or F-18 FDG (fluorodeoxyglucose), or to quantify receptors using specific radioligands.

Early PET studies with FDG reported a relative hypometabolism in frontal cortex, a finding consistent with even earlier single-photon-emission computerized tomography (SPECT) blood flow studies [86, 87]. Subsequent PET/FDG studies in schizophrenia produced inconsistent detection of frontal cortex hypometabolism, with some studies continuing to find it [88], others reporting no change in the measure [89], and still others finding frontal hypermetabolism [90]. However, a recent meta-analysis suggests that hypofrontality at rest is found in schizophrenia [91]. Still, there exists two potential confounds. First is antipsychotic drug treatment since neuroleptics are known to reduce neuronal activity in the frontal cortex. Second, deficit symptoms in schizophrenia are associated with reduced frontal cortex activation and could serve to confound observations. Studies have certainly confirmed frontal cortex alterations in schizophrenia with variable, complex, and still incompletely understood characteristics.

7.3.1.3.1 Psychosis Neural Network. Some investigators have suggested altered functional connections between brain regions as the cause of abnormal rCBF patterns seen in schizophrenia [89, 92, 93]. We and others have found evidence for limbic abnormalities in schizophrenia both at rest [89] and with cognitive challenge [94–96]. FDG PET scans at rest showed glucose utilization (rCMRglu) differences between normal and positive-symptom schizophrenia groups in the anterior cingulate cortex (ACC) and the hippocampus (HC) [89]. A follow-up study of similar design tested the regional associations of positive symptoms and showed that as long as patient volunteers were medication free, there was a significant association between rCMRglu in the limbic cortex (ACC plus HC) and the magnitude of positive symptoms in the illness; this correlation was not obtained when patients were medicated or with other symptom domains [97]. These studies allow us to speculate that it is the limbic cortex which is associated with the positive symptoms of the illness, while the PFC may support negative and/or cognitive symptoms. Blood flow changes in the anterior cingulate and adjacent medial frontal cortex also correlate with induction of positive symptoms with the NMDA antagonist ketamine [98]. PET scanning in hallucinating schizophrenic persons is associated with activations in several brain regions, including the medial prefrontal cortex, left superior temporal gyrus (STG), right medial temporal gyrus (MTG), left hippocampus/parahippocampal region, thalamus, putamen, and cingulate [99, 100].

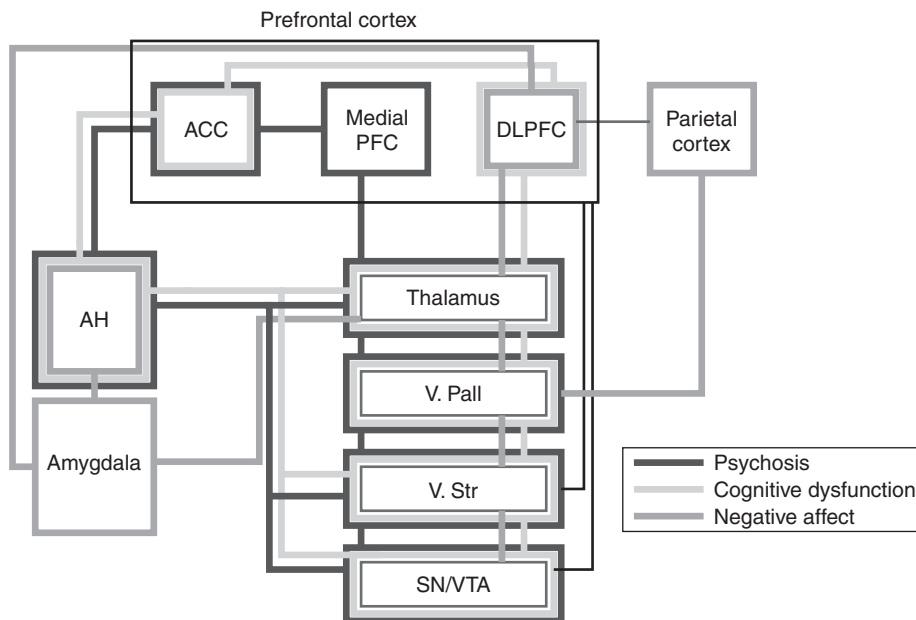


Figure 7.1 Schematic of hypothetical neural systems underlying the three symptom domains: psychosis, cognitive dysfunction, and negative affect. AH = anterior hippocampus; ACC = anterior cingulate; PFC = prefrontal cortex; DLPFC = dorsolateral prefrontal cortex; V. Pall = ventral pallidum; V. Str. = ventral striatum; SN/VTA = substantia nigra/ventral tegmental area. (See color insert.)

These studies provide clues to the anatomic structures that may be involved in a “psychosis neural circuit”. Limbic regions, in particular, are frequently implicated in these *in vivo* studies. Taking these data along with anatomic considerations, we postulate that a neural system for the psychosis cluster in schizophrenia consists of the anterior hippocampus, anterior cingulate, medial PFC (BA 32), thalamus, ventral pallidum striatum, and SN/VTA (see Fig. 7.1). We postulate that the core pathology lies in the hippocampus, resulting in hippocampal dysfunction affecting other regions in the network (e.g., the ACC and medial PFC).

7.3.1.3.2 Negative Affect Neural Network. Brain activation patterns associated with negative symptoms have been studied. Hypoactivation of the frontal lobe is seen with increased negative symptoms in schizophrenia [68, 101–104]. Decrease in rCBF is observed in the prefrontal and parietal cortex among patients exhibiting negative symptoms [89, 105–107]. It is interesting to note that the DLPFC and parietal cortex have dense reciprocal interconnections [108]. Another study in predominantly negative-symptom patients implicates the medial prefrontal, dorsolateral, and prefrontal cortices [109]. Lower activity was also noted in the thalamus [89], in particular the mediodorsal nucleus of the thalamus [110]. The amygdala, a key component in the circuit of emotion, is implicated in emotional processing in schizophrenia [111]. The neural system we propose for the negative-symptom cluster includes the DLPFC, parietal cortex, amygdala/anterior hippocampus, thalamus, ventral pallidum, striatum, and SN/VTA. Of interest, deficit-symptom persons with

schizophrenia exhibit greater impairment in cognitive performance [112, 113] that may reflect overlapping systems.

7.3.1.3.3 Cognitive Deficit Neural Network. In studies of verbal fluency [114] and semantic processing [115], network analysis reveals a functional disconnection between the anterior cingulate and prefrontal regions of schizophrenic subjects. Frontal lobe functional connectivity is abnormal in the schizophrenic subjects even though they had significantly activated the regions and their behavior on the tasks was not impaired. These findings suggest that the abnormalities seen in the frontal lobes of schizophrenics may be due to a problem of integration across regions and not a single regional abnormality. Functional MRI studies using cognitively demanding tasks, such as working memory tasks, produce diverse results. Manoach et al. [116, 117] used the Sternberg item recognition working memory paradigm, which required the subjects to remember either two or five digits. Unlike many PET studies of working memory, they found an increase instead of a decrease in prefrontal rCBF in the schizophrenic volunteers as compared to the normal controls. Callicott et al. [118] using the N-back task and Stevens et al. [119] using the word and tone serial position task found decreases of rCBF in inferior frontal regions of schizophrenic subjects. The task performance of the schizophrenic subjects was significantly worse on the N-back and word serial position task but was matched on the tone task. Research has shown that although rCBF increases in prefrontal regions with greater working memory demands, if working memory capacity is exceeded, the activation decreases [120]. Manoach suggests that the discrepant findings in schizophrenia may be explained by an overload of working memory in schizophrenic subjects for some tasks. In separate studies, disorganization was associated with flow in anterior cingulate and mediodorsal thalamus [106] while apomorphine, a dopamine agonist that has antipsychotic properties, normalizes anterior cingulate blood flow of schizophrenic persons during verbal fluency task performance [121]. We propose that a neural system for cognitive deficits involves the DLPFC, anterior hippocampus, anterior cingulate, thalamus, ventral pallidum, striatum, and SN/VTA (Fig. 7.1).

7.3.1.3.4 Neuroreceptor Imaging. Neuroreceptor PET and SPECT imaging allow direct assessments of receptor density and estimations of neurotransmitter release in the living brain. Human brain imaging ligand studies suggest abnormalities in D_1 receptor density in the frontal cortex of persons with schizophrenia [122, 123] leading to speculation that an agonist at the D_1 receptor may be therapeutic in treating cognitive dysfunctions in schizophrenia [124]. Imaging studies with D_2 dopamine receptor ligands reported increases in D_2 family receptors in neuroleptic-naïve and neuroleptic-free schizophrenia [125], and a later report suggested its presence in psychotic nonschizophrenics [126]; however, subsequent studies using various other D_2 ligands and replications with the initial ligand have been unable to replicate this finding [127–130]. All schizophrenic individuals do not have increased D_2 family receptors in the striatum, but an alteration in D_2 density may be characteristic of a subgroup of schizophrenic patients, perhaps those with a long duration of illness or other special clinical characteristics [131]. The question remains whether D_2 family receptors are elevated in a subgroup of schizophrenic patients or reflect a confound of medication effect in the initial report. More recently, Laruelle et al. [132] measured

dopamine release into the synapse using SPECT or PET imaging with low-affinity dopamine receptor ligands. They report that persons with schizophrenia have an increased release of dopamine in the striatum during the acute phases of their illness in response to amphetamine challenge compared to healthy controls [133]. A significant correlation was seen with dopamine release in the striatum and psychosis but not negative symptoms. Increased release seems not secondary to chronic antipsychotic treatment, since augmented release also occurs in first-episode patients and some family members [134]. This increase in dopaminergic tone in the striatum appears, at least in part, to be under glutamatergic regulation [135, 136]. Imaging glutamate receptors is of keen interest but is hampered by the difficulty in synthesis of such ligands, although efforts are underway [137].

Functional imaging studies have provided the most direct data on neural substrates associated with the symptom clusters while neuroreceptor imaging studies allow in vivo determination of specific receptor abnormalities and indirect measures of neurotransmitter release in specific brain regions. These studies in schizophrenic volunteers can provide in vivo data on molecular abnormalities in specific neural systems. In this section, we have proposed neural systems that may underlie each of the symptom clusters.

7.3.2 Human Post Mortem

7.3.2.1 Structural. Schizophrenia lacks identifiable neuropathological lesions such as occur in Parkinson's disease or Alzheimer's dementia. The pathology is subtle. Numerous studies utilizing a variety of techniques cite decreases in cortical thickness, abnormalities of cell size, cell number and packing density, area, neuronal organization, gross structure, and neurochemistry [138–140]. These data, however, have not been consistently replicated across laboratories, making it difficult to build a consistent story across all of the findings. This may be due to differences in patient populations, stage of illness, and medication status, although possible confounds of tissue artifact, agonal state, chronic drug treatment, lifelong-altered mental state, and relevant demographic factors must always be considered in evaluating postmortem brain tissue studies.

7.3.2.2 Neurochemical. Neurochemical studies began in schizophrenia at a time when scientists were anticipating a single protein defect. While no single defect has been found, investigators interpret studies in a broader systems context. Nonetheless, findings are often still organized by neurotransmitter system.

Studies of biochemical markers of the dopamine system in schizophrenia were stimulated by the early pharmacological observation that blockade of dopamine receptors in the brain reduces psychotic symptoms [141]. The hypothesis derived from this observation that dysfunction of the dopaminergic central nervous system (CNS) either in whole or in part accounts for psychosis in schizophrenia has been explored in all body fluids and in various conditions of rest and stimulation over the last half century [142, 143], with little real support except for the recent imaging studies that show higher occupancy of D₂ receptors by dopamine (DA) in patients with schizophrenia [144] and changes in DA release in acute illness phases [134].

More recently, because of its ubiquitous and prominent location in the CNS and because the antiglutamatergic drugs phencyclidine (PCP) and ketamine cause a

schizophrenic-like reaction in humans, the glutamate system has become a focus of study. Several studies have examined ionotropic glutamate receptor subtypes [145]. Most studies have focused on the mesial temporal lobe and, in general, report abnormalities in α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA), and NMDA receptor expression at the messenger RNA (mRNA), protein, and ligand binding level. In the prefrontal cortex, results are inconsistent, although recent studies report AMPA abnormalities [146, 147]. Changes are also reported in ionotropic receptor [148] and PSD 95 expression [149] in the thalamus. Few studies have examined the metabotropic glutamate receptors (mGluRs) in schizophrenia. Group II (mGluR2 and 3) receptors are implicated in animal [150] and human [151] studies. *N*-acetylaspartylglutamate (NAAG), an endogenous agonist of mGluR3 [152, 153], and its metabolic enzyme [154, 155] are abnormal in schizophrenia. Additionally, mGluR3 may be a risk gene for schizophrenia [156].

Evidence of γ -aminobutyric acid (GABAergic) involvement is found in reduced expression of presynaptic markers in subpopulations of interneurons in the frontal cortex and the hippocampal formation [157, 158]. GABAergic neurons can be defined by the presence of one of three calcium binding proteins, namely parvalbumin, calretinin, and calbindin. The most characteristic morphological types of neurons that express parvalbumin are the large basket and chandelier cells [159]. GAD 67 and GAT1 are decreased in the parvalbumin-expressing prefrontal interneurons [160].

The affinity of newer antipsychotic drugs for serotonergic receptors has raised speculation over the role of this neurotransmitter system in the treatment and perhaps in the pathophysiology of the illness. Years ago, serotonin was hypothesized to be central to the pathophysiology of schizophrenia, because of the psychotomimetic actions of serotonergic drugs, such as lysergic acid diethylamide (LSD) [161]. Postmortem studies have failed to find consistent change in measures of the serotonin system in schizophrenia, including in receptors (in vivo and postmortem) or in metabolites [162]. Since serotonin has been shown to modify dopamine release in striatum [163, 164], the augmented antipsychotic action of the new drugs may be mediated through modulation of dopamine release into the synapse. Indeed, drugs without any dopamine receptor affinity but with only 5-HT_{2A} receptor antagonism do behave as antipsychotic drugs in animal models and show antipsychotic activity in humans [165]. Since the serotonin system has diverse receptors and functions, it is not surprising that this aspect is not yet fully explicated.

Cholinergic neurotransmission, integral to cognition and memory, may be dysfunctional in schizophrenia. Clinically, it is well known that schizophrenic patients have a much higher incidence of cigarette smoking [166]. Although "control" smokers exhibit an upregulation in nicotinic receptors [167, 168], decreased levels of nicotinic and muscarinic receptors are reported in the hippocampus frontal cortex, thalamus, and striatum in schizophrenia [169].

Molecular abnormalities are found in a number of anatomic regions and in several neurotransmitter systems in the neuropathology of schizophrenia. Abnormalities in molecular targets should be examined in terms of pathways (not only neurotransmitter pathways) affecting circuit function. Additionally, identification of primary pathology from epiphenomenon is essential. For example, neurotransmitter systems are dynamic and disruption of one system would lead to compensatory mechanisms in other relevant pathways. In general, a strategy to follow once a positive finding is

made would be to confirm the finding is real and not an artifact, replicate the finding in another cohort, and determine if the molecular abnormality is part of the primary pathology. Converging data from in vivo human studies, post mortem human studies and animal model studies would provide clues to the primary pathology.

7.3.3 Genetics and Phenotypes

Schizophrenia is heritable but does not follow a simple pattern. Multiple susceptibility genes, each of small effect, are believed to exist for schizophrenia. Replicated linkages to several chromosomal regions have been made, including to 8p, 22q, 2, 3, 5q, 6p, 11q, 13q, and 20p, and there are several genes within these regions that have been associated with the illness, including neuregulin (*NRG1*) [170], dysbindin (*DTNBP1*) [171], *G72* [172], D-amino-acid oxidase (*DAAO*), regulator of G-protein signaling 4 (*RGS4*) [173], proline dehydrogenase (*ProDH*) [174], catechol-*O*-methyl transferase (*COMT*) [175, 176] and metabotropic glutamate receptor 3 (*mGluR3*) [156]. Each of these genes codes for a protein that has been speculatively linked with a purported illness mechanism [177, 178], but no clear disease pathophysiology has yet emerged. The goal of confirming a susceptibility gene for schizophrenia is to acquire molecular information about disease mechanisms, which could potentially lead to a broader understanding of the illness and be a basis for novel treatment development.

Investigators argue that an important impediment in understanding the neurobiology of schizophrenia is disease heterogeneity. This may account for the lack of firm knowledge of disease pathophysiology that is the biggest impediment to progress in therapeutics. Therefore, investigators have been attempting to define more homogeneous phenotypes of schizophrenia in persons with the illness and in family members ("endophenotypes") to test these genetically [17]. The features most often used to develop phenotypes in the illness are neurocognitive characteristics, eye movements [179–181], prepulse inhibition (PPI) [182, 183], evoked potential [184], and in vivo brain imaging features (reviewed in Gottesman and Gould [185]). These are spontaneous behaviors of the brain occurring in response to external cues that have a known neural anatomy, and hence may be more direct reflections of neural pathology [186]. The ability of some probands (60–70%) with schizophrenia to follow a smooth pendulum movement with their eyes is deficient [187]. Instead of describing smooth movements following a pendulum stimulus, some show jerky and irregular (delayed and catch-up movements) tracking patterns. Also, antisaccade eye movements (those directed away from a stimulus) are also abnormal in persons with the illness [187, 188]. PPI is a normal phenomenon evident across all sensory modalities, where a small initial ("pre") stimulus decreases the electrophysiological response to a second higher intensity stimulus. In schizophrenia, many probands show abnormal PPI, as do unaffected family members. The neural systems influencing both oculomotor movements and PPI have been well described in the animal and are believed to be highly conserved in the human [189, 190]. P50 is an electrophysiological measure produced when two equal auditory stimuli are presented 500 ms apart and their evoked potential is measured. Healthy persons show a reduced response (in amplitude) to the second signal whereas persons with schizophrenia (estimated at 80%) show less or no suppression.

These data will help identify candidate genes and allow rational selection of molecular targets for further investigation. Although genetic involvement in schizophrenia is

certain, genetic makeup alone does not predict schizophrenia. Also schizophrenia typically does not manifest until the second or third decade of life, implying an interaction between genotype and other factors prior to onset of the illness. It is possible that as the brain matures (e.g., during adolescence) neural networks in the brain are developing and becoming established, a time when molecular abnormalities may become apparent.

7.4 ANIMAL MODELS

Schizophrenia, as we know it, is a uniquely human disorder; therefore creating an exact or full animal model of this illness may not be possible. It is, however, possible to create models to study aspects of disease etiology or pathophysiological mechanisms and to create models for target symptom areas. Approaches include genetic manipulations, pharmacological and environmental manipulations, and discrete anatomic lesions. These animal models produce symptoms that qualify as belonging to one or more of the three symptom clusters.

7.4.1 Cognitive Deficit Model

7.4.1.1 *Working Memory Deficits.* The limited cognitive capacity of animals limits the design of cognitive tasks. Olton and Samuelson [191] devised a classic task for assessing memory in the rodent, the radial arm maze. Delayed alternation problems capitalize on the rats' tendency to choose alternative maze arms or locations when rats are reexposed to an apparatus, the most common version is the T maze. Delayed nonmatching to sample tasks require a rat to remember a stimulus over a delay in which that stimulus is no longer present. Nonhuman primate working memory models include spatial delayed response, delayed match to sample, and attentional set-shifting tasks [192].

7.4.1.2 *Declarative Memory Deficits.* Explicit or declarative memory is abnormal in schizophrenia [193–195]. This is hippocampal dependent and has been examined in animal models. The role of the hippocampus in learning and memory tasks that encourage animals to compare and contrast odor items as they learn about them and to encode both direct and indirect relations among odors has been described [196]. Transitive inference (TI), a task of the ability to infer a relationship between items that have not been presented together, is abnormal in schizophrenia [197] and can be tested in rodents [198].

7.4.1.3 *Attention Deficits.* The continuous performance test (CPT) developed by Rosvold et al [199] is a paradigm used to test attention in schizophrenia. Subjects continually monitor the location of a brief visual target in one of five spatial locations that occur randomly [200], providing a measure of visuospatial attention. An analogous test for use in rodents is the five-choice serial reaction time task [201]. This test has been used to examine effects of drugs and neuroanatomical lesions on various aspects of attentional performance, including selective attention, vigilance, and executive control [202, 203].

7.4.1.3.1 Social Interactions. Social withdrawal is a significant feature in schizophrenia. Dyadic encounters have often been used to investigate social behavior in rats [204, 205]. Social recognition is assessed by quantifying the duration of social investigation during subsequent exposures to the same individual. Reexposure is characterized by a shorter investigation time. This foreshortened time is taken to represent the social recognition. Social cognition is a particular aspect of learning and memory that is selectively impaired in schizophrenia [206]. In rats, ketamine disrupts social learning, an effect that can be reversed by antipsychotic treatment [207]. The prairie vole provides another model to study social interactions. These rodents are highly social, form selective, enduring pair bonds with one mate [208, 209], and are amenable to experimental manipulation.

7.4.2 Genetic Models

A number of mutant mice for several neuroreceptors have been developed, including those for NMDA receptor subunits [210], mGluR1 [211], mGluR5 [212], dopamine receptor subtypes [213], and adrenergic α_{2A} receptors [214]. Of these, the most interesting mutant mice are those deficient in NMDA glutamate receptors generated by targeted mutation of the crucial NR1 subunit gene [210]. NR1 knockout mice display increase in activity and stereotypies that were attenuated by antipsychotic treatment. They also exhibit negative symptoms evidenced by the decrease in social interactions. These behaviors are similar to those observed in the PCP or MK-801 treated animals. Mutants for the transcription factors NPAS1 and NPAS3 have been reported to result in PPI deficits, impaired social recognition, and locomotor abnormalities [215].

7.4.3 Psychosis Model

Pharmacological models designed to perturb a particular neurotransmitter system have been described. These models induce “psychosis” but not exclusively:

1. *Glutamatergic Models* NMDA receptor antagonists, such as PCP and ketamine, induce psychotic symptoms in healthy humans [216–219] and exacerbate psychotic symptoms in patients with schizophrenia [220]. It is also found to disrupt PPI, an effect that can be reversed by atypical antipsychosis [221, 222]. In rodents, PCP administration induces behavioral activation observed as hyperlocomotion and stereotypies (positive symptoms) [223–229], impaired performance on learning and memory processes (cognitive deficit cluster) [230], and diminished social interactions (negative-symptom cluster) [229–231]. These effects can be reversed by mGluR2/3 agonists [150, 232].

2. *Dopamine Agonist Models* Hyperactivity of the mesolimbic dopaminergic system and hypoactivity in the frontal cortex have been postulated as producing positive and negative symptoms, respectively, in schizophrenia [233]. Similar to the effects of NMDA antagonists, amphetamine induces psychotic reactions in normal individuals and exacerbates symptoms in schizophrenic patients [234, 235]. Negative symptomatology, however, is not reproduced by amphetamine administration [236].

3. *Cannabinoid Model* Administration of Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the major active component in cannabis, transiently increases positive and negative symptoms and cognitive deficits in healthy human volunteers and subjects with schizophrenia [237]. The *val/met* functional polymorphism in the *COMT* gene influences the development of psychosis in adolescent marijuana users [19]. Carriers of the *val158* allele, but not the *met* carriers, were much more likely to develop symptoms of psychosis. This suggests an interaction between genetic predisposition and environmental factors. In rats, Δ^9 -THC influences cognitive and behavioral functions in rats [238–241].

7.4.4 Neurodevelopmental Model

7.4.4.1 Disruption of Neurogenesis. This model aims to disrupt normal cell division and maturation at specific gestational time points in an attempt to reproduce abnormalities found in schizophrenia. Prenatal injection of the mitotic inhibitor methylazoxymethanol (MAM) at day E17 produces behavioral changes mimicking the positive and negative symptoms of the disorder. Aberrant cell migration in the hippocampus and a disrupted laminar pattern in the neocortex associated with deficits in working memory [242] and other specific cognitive deficits [243] are observed in these animals. Another approach is the irradiation of monkeys in midgestation which produces cortical abnormalities reminiscent of schizophrenia [244]. Behavioral tests have not yet been reported for these monkeys.

7.4.4.2 Lesion Models. Neonatal ventral hippocampal lesions in rats have been proposed as an animal model [245]. This lesion interferes with development of the hippocampus and its associated connections with other brain regions such as the prefrontal cortex. These animals exhibit some behavioral and molecular changes observed in schizophrenia [246–248]. Lesions in other brain structures like the medial prefrontal cortex [249] have been described.

Animal models in schizophrenia have been criticized as not being definitive. While it may not be possible to reproduce the full syndrome in animals, it does not mean that animal models are not useful or informative about the discrete characteristics they demonstrate. It would be ideal if each symptom cluster and associated neural circuit could be examined separately. Each circuit, however, is not discrete and overlap is present, allowing one circuit to influence the others. Using a number of different animal models targeting specific proteins, pathways, anatomic regions, or circuits and examining the converging data will provide the most useful data.

7.5 CONCLUSION: HOW FAR HAVE WE COME AND WHAT ARE THE REMAINING QUESTIONS?

Schizophrenia is a complex disease most likely associated with multiple etiologies. Clinically, the illness can be identified by its typical presentation, but researchers endeavor to distinguish subgroups within the diagnosis “schizophrenia” in an effort to identify unique pathophysiological mechanisms. The phenotypes of schizophrenia, defined by symptoms, psychological, electrophysiological, biochemical, or physiological

(i.e., rCBF) characteristics, are being proposed. The sorting of these phenotypes into biologically meaningful groups is ongoing. In brain imaging studies, distinct anatomic substrates for the three symptom clusters (psychosis, cognitive dysfunction, and negative affect) suggest the presence of specific neural networks for each cluster. It is possible that endophenotypes identified by electrophysiological or neurocognitive means also share neural network dysfunction.

A shift in conceptual framework from defects in specific proteins to defects in neural networks may represent a biologically relevant mechanism useful in investigating the pathophysiology of schizophrenia. This will require identification of relevant neural systems and an understanding of the dynamics of the systems. Then such systems could be used as a model to test function—what are the effects of disrupting one part of a network? Are there compensatory mechanisms that come into play? Are there regions that are crucial to the functioning of the system? How can we modulate the system in the diseased state to make it more efficient? The system's neuroscience approach in schizophrenia is at its very early stages of conceptualization. As an initial formulation, we propose neural networks for each of the symptom domains (Fig. 7.1). This formulation proposes that the core pathology in the hippocampus influences function of networks involving distinct cortical regions and subcortical structures.

Whether the symptom domains are manifestations of a single disease pathophysiology perturbing the neural networks or are each a partially independent disease construct remains unknown. However, heterogeneity is more often presumed about the illness, certainly with respect to etiology. An integral part of determining function of affected neural systems is understanding the molecular composition of the connections in the network. The primary molecular lesion of schizophrenia or of any one of its subgroups has not yet been identified. Whether productive leads will derive from clinical pharmacology, genetics, imaging, or phenotyping or perhaps from all is a speculation. It is the kind of discovery that will be used to target novel drug discovery. Without known molecular targets, therapeutics can advance only by serendipity, chance, or modifications of existing treatments. Hope for identifying the pivotal molecular targets for schizophrenia rests on the application of modern concepts and techniques to clearly diagnosed and characterized populations of persons with schizophrenia. There are therapeutic implications to heterogeneity: Does one treatment exist for schizophrenia or are there several symptom or syndrome-specific treatments for the illness? This question remains open, but taking clues from other illness, one would guess that several treatments will emerge.

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8

DOPAMINE AND GLUTAMATE HYPOTHESES OF SCHIZOPHRENIA

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8.1	Introduction	283
8.2	Dopamine Hypothesis	284
8.2.1	History	284
8.2.2	Pathological Evidence	285
8.2.3	Imaging Evidence	285
8.2.4	Genetic Evidence	286
8.2.5	Pharmacological Evidence	287
8.2.6	Evolution of Dopamine Hypothesis: Cortical Versus Striatal Dopamine	287
8.3	Glutamate Theory of Schizophrenia	289
8.3.1	History	289
8.3.2	Pathological Evidence	291
8.3.3	Imaging Evidence	292
8.3.4	Genetic Evidence	293
8.3.5	Pharmacological Evidence	294
8.4	Consolidating Glutamate and Dopamine Hypotheses of Schizophrenia	295
	References	297

8.1 INTRODUCTION

Discovery of the first generation of antipsychotic drugs in the 1950s is often credited with the birth of neuronally based approaches to explain the etiology and the pathophysiology of schizophrenia. While at the time this discovery presented a major conceptual shift from the Freudian psychosocial approach to explaining schizophrenia, it is important to note that the pre-Freudian ideas about schizophrenia (*dementia praecox*) were eerily similar to our current concepts and understandings of the disease, specifically, that the etiology is dependent on genetic predisposition [1] and that the primary pathophysiology may involve cortical dysfunction [2, 3]. This is apparent in many texts and research papers written on mental illness at the turn of

the century where, in describing the “etiology” of the disease, statements like “in the causation of dementia praecox the hereditary factor is the most important: the other factors are, for the most part, contributory or excitatory” [4, p.16] or “the importance of heredity as a factor concerned in the etiology of schizophrenia has long been recognized” [5, p.77] were the norm. Similarly, the leading pathological theories about schizophrenia by Mott, Kraepelin, and Alzheimer included gliosis of the cortex and dissociated activity of the afferent and efferent neurons causing an imbalance of “pyramidal” activity [6, 7]. Given this historical context, our approach in this chapter on dopamine and glutamate hypotheses of schizophrenia has been to focus on the evolution of these theories. Recent advances in postmortem, imaging, and genetic fields clearly indicate that schizophrenia is not caused by abnormalities in a single gene, a single neurotransmitter or receptor, or a single brain region. Dopamine and glutamate hypotheses of schizophrenia, although narrowly defined at conception, have evolved to accommodate these most recent neuroscientific findings and thus remain two of the most influential theories in the field.

8.2 DOPAMINE HYPOTHESIS

8.2.1 History

The dopamine hypothesis of schizophrenia, in its original form, stated that a hyperactive dopamine transmission is responsible for the psychotic features of the disease [8]. This theory was consistent with the then newly emerged concept that neurochemical imbalances in the brain may be responsible for major psychiatric disorders [9–12]. A few years earlier, clinical studies with chlorpromazine [13] and reserpine [14, 15] had demonstrated, for the first time, the effectiveness of pharmacotherapy in treating psychotic symptoms of schizophrenia. Subsequent animal studies showed that reserpine, which was later found to inhibit the vesicular monoamine transporter protein (VMAT), increased tissue levels of serotonin and norepinephrine (these studies did not assess the levels of dopamine as dopamine neurons had not yet been discovered). These findings led to the hypothesis that schizophrenia was associated with a state of monoamine deficiency and that neuroleptics ameliorated this condition by increasing the release of serotonin and norepinephrine [9]. Upon discovering the dopamine neurons, Carlsson and Lindquist found that chlorpromazine and another antipsychotic drug haloperidol increased the metabolite levels of dopamine, suggesting that these drugs increase the turnover rate of this neurotransmitter [8]. The authors then proposed that this increased turnover was a *compensatory* mechanism that was secondary to the blockade of dopamine receptors. The idea that inhibition of dopamine receptors, as opposed to enhancing monoamine release, mediates the actions of neuroleptics gained acceptance after the identification of two different subtypes of dopamine receptors, D₁ and D₂, and became the prominent theory of the field after the seminal finding that all antipsychotic drugs block the dopamine D₂ receptors with affinities that correlate with their clinical efficacy [16, 17]. Concurrent with these studies, it was found that psychostimulants that increase dopamine neurotransmission, either through release of endogenous dopamine [18–20] or through direct activation of dopamine receptors [21–23], had psychotomimetic properties in nonschizophrenics and exacerbated symptoms in patients with schizophrenia. Together, these findings lend a great

deal of support for Carlsson and Lindquist's initial idea that antipsychotic drugs help to alleviate a hyperactive dopamine system and led to intense efforts to find direct evidence for a disrupted dopamine transmission in schizophrenia. However, the substantial body of research, spreading over five decades, which has been undertaken to address this fundamental issue has not yet provided direct evidence that over-activation of the dopamine system accounts for the spectrum of symptoms that are associated with schizophrenia. But these studies have found many interesting, albeit subtle, changes in some dopamine systems in schizophrenia which have prompted the formulation of more elaborate ideas about dopaminergic abnormalities in schizophrenia.

8.2.2 Pathological Evidence

Over 50 years of intense research has failed to show postmortem dopamine-related abnormalities that would be consistent with a hyperactive dopamine state in schizophrenia. Although several studies have reported an increase in striatal D₂ receptor density in postmortem schizophrenic tissues [24–27], the interpretation of these results is confounded by the fact that chronic antipsychotic treatment can upregulate D₂ receptors [28]. Other dopamine-related findings in postmortem schizophrenic brains include (1) a lack of change in striatal and prefrontal cortex (PFC) D₁ receptor density and messenger RNA (mRNA) levels [29–34]; (2) a reported increase in striatal D₃ receptor number [35] that was not associated with change in striatal D₃ receptor mRNA levels [30]; (3) an increase in striatal D₄ receptor density [36–38] that was not confirmed by other studies [39–41]; (4) a lack of change [42, 43] (but also see [44]) in the expression of D₄ mRNA levels in the PFC; (5) a lack of change in striatal dopamine transporter (DAT) density [33, 45–47]; and (6) unaltered activity of striatal dopamine-related enzymes such as tyrosine hydroxylase (TH), dopamine β-hydroxylase, and catechol-*O*-methyl transferase (COMT) [48]. Moreover, limited and inconsistent changes in the concentration of tissue dopamine and its metabolites have been reported in schizophrenic brains [49, 50]. Collectively, these negative findings have engendered speculations that dopaminergic abnormalities in schizophrenics may involve subtle and activity-dependent abnormalities that cannot be detected in postmortem tissues.

8.2.3 Imaging Evidence

New evidence from in vivo imaging studies has been instrumental in advancing our knowledge of the dynamics of the dopamine systems in schizophrenia. In general, imaging studies have the advantage of allowing investigators to control for the effects of treatment, to correlate the abnormalities with clinical symptoms, and to study the in vivo functional dynamics of dopamine release. For example, imaging studies in untreated drug-naïve schizophrenic patients have addressed the confounding effect of previous neuroleptic treatment on striatal dopamine receptor density. While two studies reported increased striatal D₂ receptor density in schizophrenia [51, 52], a meta-analysis of 17 studies reported a relatively small (12%) increase in the density of these receptors [53], leading to the consensus that it is unlikely that such an inconsistent and weak effect on D₂ receptor density would be the primary cause of schizophrenic pathology. The imaging findings with other dopamine receptor

subtypes have also been inconsistent. For example, a high-profile study reporting an increase in the density of D₁ receptors in the PFC of schizophrenics [54] subsequently was not replicated [55]. On the other hand, interesting and consistent results have emerged from investigations of the functional dynamics of dopamine release in schizophrenia. For example, positron emission tomography (PET) studies following administration of radiolabeled dopamine substrates such as dopa or fluorodopa have shown that patients with schizophrenia have a higher rate of dopamine synthesis than normal controls [56–59]. Others, using PET and single-photon-emission computerized tomography imaging during an amphetamine challenge to assess the release of endogenous dopamine, have found a higher level of dopamine release in response to amphetamine in patients with schizophrenia compared to normal controls [60, 61]. Interestingly, this effect was associated with acute exacerbations of psychotic symptoms but could not be detected when patients were in a phase of symptom stabilization [62]. A recent report of increased baseline occupancy of striatal D₂ receptors in schizophrenics is of special interest [63], though this finding remains to be replicated. Together, these recent findings have strengthened the link between dopaminergic hyperactivity and positive symptoms of schizophrenia [50, 64] but have not established a primary role for dopamine abnormality in this disorder. Another important PET study measured D₁ receptor availability in drug-naïve or drug-free patients and reported that D₁ receptor antagonist binding potential was significantly elevated in dorsolateral PFC of patients with schizophrenia [65]. This measure demonstrated an increased availability of D₁ receptors which would result from reduced, as opposed to increased, dopaminergic neurotransmission. Implications of this dichotomy are discussed later.

8.2.4 Genetic Evidence

Schizophrenia is a multifactorial disease with a complex mode of inheritance [66]. There has been limited convergence of evidence on dopamine-associated genetic loci that may be associated with schizophrenia. Positive reports of D₃ receptor gene polymorphism associated with schizophrenia [67, 68] were not confirmed in other association or linkage studies [69, 70]. Nonetheless, a recent meta-analysis of genetic studies has shown a small degree of association for the D₃ receptor gene [71]. Linkage studies involving genes for other dopamine receptors as well as dopamine related proteins have been mostly negative or ambiguous [69, 72]. One exception, however, involves the gene that encodes for the enzyme COMT. This is an extracellular degradative enzyme that converts dopamine to its aldehyde derivative and, therefore, plays a critical role in regulating the extracellular levels of dopamine [73]. The COMT gene is on 22q11, which is considered a chromosomal “hot spot” for genes that are associated with increased vulnerability to develop schizophrenia [74]. A common functional polymorphism on this gene (Val¹⁰⁸/¹⁵⁸Met) accounts for up to a fourfold variation in enzymatic activity and the subsequent dopamine metabolism. In patients with schizophrenia this polymorphism may predict 4% of the variance in performance on cognitive tasks, such as the Wisconsin card sort task (WCST), that are dependent on the proper functioning of cortical dopamine [75]. Subsequent studies have replicated the effects of COMT genotype on PFC cortical function [76, 77]. However, a recent meta-analysis failed to show a significant association of COMT polymorphism with schizophrenia [71]. While this COMT polymorphism may not

have a substantial influence on the pathophysiology of schizophrenia, its convincing influence on cognitive function suggests that it may contribute to poor cognitive capacity of some patients with schizophrenia [2].

8.2.5 Pharmacological Evidence

In the absence of direct evidence for dopamine hyperactivity in schizophrenia, the pharmacological profile of antipsychotic drugs continues to provide the strongest support for a hyperdopaminergic state in schizophrenia. Imaging studies have established a link between D_2 receptor occupancy and clinical efficacy of antipsychotic drugs (see [78, 79] for review). While all clinically efficacious antipsychotic drugs lead to significant D_2 receptor occupancy, the imaging studies have failed to establish a direct relationship between the degree of striatal D_2 receptor occupancy and the clinical efficacy of antipsychotic drugs [80–82], primarily because ample D_2 receptor occupancy is observed in patients who do not respond to conventional antipsychotic treatment [81]. This fact has reinforced the idea that D_2 occupancy may be necessary but not sufficient for treatment of schizophrenia and has led to suggestions that co-occupation of D_2 with other receptors such as the dopamine D_3 [83] or D_4 [84, 85], the serotonin 5-HT₂ [86] or 5-HT_{1A} [87], and the α -noradrenergic [88] receptors may lead to more efficacious treatment. On the other hand, Kapur and Seeman [89] have argued that optimal modulation of D_2 receptors involving factors such as rate of dissociation, and not complementary actions on other receptors, is the key to clinical efficacy of atypical antipsychotics. In addition to D_2 receptor antagonism, other manipulations of dopaminergic transmission have been explored as potential therapeutic strategies in schizophrenia. Some researchers have attempted to develop a therapeutic strategy based on pure D_4 receptor antagonism [90] primarily because the atypical antipsychotic drug clozapine has preferential affinity for D_4 over D_2 receptors. However, clinical trials with two selective D_4 blockers, sonopiprazole and L-745,870, have been disappointing [90, 91]. Others have suggested that partial D_2 receptor agonists may be used as dopaminergic stabilizers to antagonize the excessive activation of the dopaminergic system without inducing a hypodopaminergic state that has been associated with serious motor and motivational side effects of current drugs [92, 93]. Preliminary studies with the partial D_2 agonist (–)-3PPP have produced positive results [94, 95]. Another drug in this class, aripiprazole, has been approved recently for clinical use and early results on the clinical efficacy and side-effect profile of this drug appear promising [96–98]. Whether this superior profile is mainly due to dopaminergic modulation remains a matter of debate because aripiprazole also modulates serotonin 5-HT_{1A} and 5-HT_{2A} receptors [99]. Interestingly, the selective dopamine D_2/D_3 receptor antagonist amisulpride was recently introduced as the first atypical antipsychotic that does not have a significant affinity for 5-HT_{2A} or other serotonin receptors. Ongoing clinical trials with this drug will allow for a better assessment of the exclusive contribution of dopaminergic effects to the therapeutic profile of antipsychotic drugs [100, 101].

8.2.6 Evolution of Dopamine Hypothesis: Cortical Versus Striatal Dopamine

An increased understanding of the principles that govern higher cognitive functions has led to formulation of more elaborate versions of the dopamine hypothesis of

schizophrenia. This progress has been made in the larger framework of a “systems neuroscience” approach to complex psychiatric disorders, where (1) distinct behavioral components of a clinical syndrome are considered and (2) relevant molecular, cellular, and circuit-based data from “normal” (human or animal) subjects are applied to define mechanisms that subserve these behavioral components. In the context of schizophrenia, applying this approach has been useful in elucidating mechanisms that may underlie the cognitive and negative symptoms of this disorder. The most notable insight has come from studies that have characterized the role of PFC in maintaining cognitive functions that are relevant to schizophrenia. In general, numerous imaging studies have shown that several subregions of the PFC are activated during performance of cognitive tasks that require working memory or set shifting [102–106]. In patients with schizophrenia, which generally exhibit impaired working memory, this task-dependent activation of cortical activity is diminished (see [73] for a review). Seminal work by Goldman-Rakic and coinvestigators has shown that a key neuronal system in the PFC for maintaining working memory is the dopaminergic projection to this region [107–110]. In particular, optimal activation of PFC dopamine D₁ receptors, a subtype that is abundant in the cortical regions [111], appears to be critical for working memory performance (see [112] for a review). These primate studies have been critical in generating the hypothesis that a deficiency in cortical dopamine transmission may underlie the cognitive deficits of schizophrenia [113, 114].

A number of clinical studies also suggest a role for cortical dopamine dysfunction in schizophrenia. For example, a direct relationship between decreased metabolic activity in PFC and reduced cerebrospinal fluid (CSF) concentration of homovanillic acid, the major metabolite of cortical dopamine, has been reported in schizophrenic patients during their performance of cognition tasks [115, 116]. Furthermore, Akil and coinvestigators [117, 118] have reported a significant reduction in the length of cortical axons that are immunoreactive for tyrosine-hydroxylase, a finding that would be consistent with reduced catecholaminergic innervation in the PFC. While the low density of D₂ receptors in the PFC has not allowed for reliable PET measurements with D₂-selective ligands, several studies have reported interesting changes in D₁ receptor occupancy in patients with schizophrenia. The first report was by Okubo et al. [54], who demonstrated a positive correlation between the increased density of D₁ receptors in PFC of patients and performance on the WCST. A more recent PET study using a superior D₁ receptor ligand has reported a contrasting finding of increased, rather than decreased, D₁ receptor binding in drug-free and drug-naïve schizophrenia patients, which showed a positive correlation with performance in a working memory task [65]. Notably, the same investigators have provided evidence from rodent experiments that the clinical findings may reflect a compensatory upregulation of D₁ receptors following sustained dopaminergic depletion, an interpretation that would fit with a deficit in PFC dopamine activity [119, 120].

Collectively, these findings have led to a more elaborate bidirectional dopamine hypothesis proposing that schizophrenia may be associated with a concomitant cortical hypodopaminergia, which presumably contributes to negative and cognitive symptoms of schizophrenia through reduced D₁ receptor activity, and a subcortical hyperdopaminergia, which leads to positive symptoms through overactivation of D₂ receptors [50, 114]. This revised theory offers a mechanism that explains not only psychosis but also the cognitive symptoms of schizophrenia and has

encouraged development of therapeutics that may specifically target these symptoms. Furthermore, this framework has prompted systems-oriented clinical research in schizophrenia which is helping to increase our knowledge of the pathophysiology of the disease. For example, Bertolino and coinvestigators [121, 122] have shown that *N*-acetylaspartate (NAA), an imaging marker of neuronal functional integrity, is negatively correlated, in dorsolateral PFC, with striatal dopamine release in schizophrenia. Meyer-Lindberg et al. [123], using double measures of regional cerebral blood flow and fluorodopa uptake in schizophrenic subjects during performance of the WCST, corroborated the relationship between hypoactivation of PFC and increased striatal dopamine utilization during a working memory task.

Pharmacological studies using dopamine receptor agonists in primates have supported the notion that activation of D₁ receptor function may be useful for treating cognitive deficits in some animal models [124–126]. While therapeutic efficacy of D₁ receptor agonists remains to be validated, this pharmacological strategy has been indirectly tested in several studies demonstrating that administration of nonspecific dopamine agonists, such as amphetamine or apomorphine, to patients with schizophrenia improves working memory performance and PFC signal-to-noise ratio [127–129].

8.3 GLUTAMATE THEORY OF SCHIZOPHRENIA

8.3.1 History

Unlike the dopamine hypothesis, which was not based on actual dopaminergic abnormalities and was formed to accommodate the mechanism of action of antipsychotic drugs, the idea of a glutamatergic hypofunction was first generated because postmortem and CSF data from patients with schizophrenia were suggestive of a dysregulated excitatory amino acid system [130, 131]. This theory, however, did not gain acceptance at the time because, first, subsequent studies did not confirm the findings of Kim and coinvestigators [132–134] and, second, our limited knowledge of the function and behavioral pharmacology of the glutamate system at the time dictated that a dysfunctional glutamate system would result in overt toxicity and developmental abnormalities as opposed to the subtle and regionally specific cellular changes that occur in schizophrenia. A major revival of the glutamate theory occurred with the discovery that the well-known psychotomimetic drug phencyclidine (“angel dust”) is a selective noncompetitive blocker of *N*-methyl-D-aspartate (NMDA) glutamate receptors [135–138]. This agent produces an acute psychotic episode with high resemblance to the spectrum of schizophrenic symptoms and profoundly exacerbates preexisting symptoms of schizophrenia in patients [139–142]. Recent clinical trials using ketamine and analogs, Federal Drug Administration (FDA)–approved blockers of NMDA receptors, have carefully characterized the profile of symptoms induced in healthy subjects by this class of compounds [143–146]. The range of symptoms produced by these agents resemble positive (delusion and hallucination), negative (avolition, apathy, and blunted affect), and cognitive (deficits in attention, memory, and abstract reasoning) symptoms of schizophrenia as well as disruptions in smooth-pursuit eye movements and prepulse inhibition of the startle response [143–150]. Moreover, administration of low doses of ketamine to patients

with schizophrenia precipitates the expression of acute psychosis incorporating symptoms that are similar in content to patients' preexisting experience [140, 151–153]. Collectively, these studies have made a strong case for an NMDA receptor deficiency in schizophrenia because they demonstrated that an NMDA receptor antagonist produces a behavioral syndrome that resembles symptoms of schizophrenia far better than dopamine agonists do [154].

NMDA receptors play an essential role in the development of neural pathways, including the critical process of the pruning of cortical connections during adolescence [155, 156], making them a likely contributor to the hypothesized developmental malfunctions in schizophrenia [157, 158]. Given the pharmacological and developmental evidence, it was suggested that a hypoglutamatergic state, probably at the level of NMDA receptors, might account for some aspects of schizophrenia, particularly the cognitive and negative symptoms that are closely associated with frontal cortical function [154, 159, 160]. During the past decade, this theory has been further modified to account for the complexity of glutamatergic transmission and its interactions with other neurotransmitter systems, especially dopamine and γ -aminobutyric acid (GABA) [161–166]. Here, we will first briefly review the main elements of the glutamate system and then discuss the evidence for a glutamatergic dysregulation in schizophrenia.

In general, glutamate receptors can be classified into two broad families: ionotropic and metabotropic receptors. Ionotropic glutamate receptors are classified into three broad subtypes according to their preferential agonists: the NMDA, kainate, and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. Binding of glutamate to these receptors stimulates Ca^{2+} entry into neurons through channels formed either by the receptor itself (as is the case with the NMDA receptor subtype) or by opening voltage-sensitive Ca^{2+} channels that are on the cell membrane. The AMPA receptors are composed of at least four subunits derived from a family of four genes termed *GluR1* to *GluR4*. Kainate receptors are thought to be composed of five identical subunits (homomers) derived from genes termed *GluR5* to *GluR7* and *KAI1* and *KAI2*. The NMDA receptor is a heteromeric complex composed of four or five subunits derived from seven genes, *NR1*, *NR2A* to *NR2D*, *NR3A* and *NR3B* [167]. The *NR1* subunit, which has several isoforms, is an obligate subunit. Nearly all neurons express AMPA and NMDA receptors, and it is estimated that glutamate ionotropic receptors mediate nearly 50% of all synaptic transmission in the mammalian central nervous system. The more recently discovered metabotropic glutamate receptors [168] have a distinct mechanism of action and use G-protein-coupled synaptic transduction mechanisms, similar to those used by the monoamine neurotransmitters, to indirectly regulate the electrical signaling. This mechanism is in contrast to rapid excitation and opening of ion channels by ionotropic glutamate receptors, which is a more suitable mechanism for pharmacological targets. At least eight metabotropic glutamate receptors have been cloned (termed mGlu1 to mGlu8). These receptors share no sequence homology with other known receptors in the nervous system, suggesting that they are members of a new receptor gene family. The eight subtypes of mGlu receptors are currently classified into three groups (termed groups I–III) based on amino acid sequence homology and transduction mechanisms. Group I (mGlu1 and mGlu5) metabotropic receptors activate the enzyme phospholipase C, which in turn results in the breakdown of membrane phospholipid to the second messengers inositol triphosphate or

diacylglycerol. Groups II (mGlu2 and mGlu3) and III (mGlu4, mGlu6, mGlu7, and mGlu8) metabotropic receptors downregulate the enzyme adenylate cyclase and result in reduced synthesis of the second messenger cyclic adenosine monophosphate (cAMP).

8.3.2 Pathological Evidence

In general, the direct evidence for glutamatergic involvement in schizophrenia is abundant and converges on several principles. First, abnormalities in various components of glutamatergic systems, including the NMDA receptor, have been reported in schizophrenic brain samples. Second, these abnormalities follow a region-specific pattern with the most impressive accumulation of evidence being so far reported from temporal and frontal cortices, limbic regions, and thalamus, with limited evidence found in striatum.

Several groups have reported altered densities of kainate and AMPA glutamate receptors in postmortem schizophrenic brains [169–173]. Harrison and coinvestigators reported decreases in AMPA receptor binding in CA3 and CA4 subfields of hippocampus [170, 172]. These findings are consistent with reports of reduced expression of GluR1 and GluR2 protein or mRNA levels in similar regions [172, 174–176]. In contrast to the temporal lobe regions, the reported changes in frontal cortex and striatal regions have been small [177]. Consistent decreases in AMPA GluR1 and GluR2 expression have also been reported in the thalamus [178]. Collectively, these data suggest that in schizophrenics' brains there are decreases in the expression of several AMPA receptor subunits and in AMPA receptor binding in the medial temporal lobe and the thalamus.

Several other studies have examined kainate receptor binding or mRNA levels [131, 179–183]. In general, these studies follow the same pattern of change as in AMPA receptor expression, suggesting reduced levels of expression in temporal lobe regions. Furthermore, a recent study reported increased transcript levels of GluR5 (a kainate subunit) in substantia nigra in schizophrenia [167].

Studies on NMDA receptor density in cortical, striatal, and temporal lobe structures have led to less consistent results (see [169] for a review). However, more recent data indicate that part of the earlier inconsistencies may be due to alterations in the subunit composition of NMDA receptors. The earliest report in the literature using [³H]MK801 described increased binding in the putamen but not in the frontal cortex or temporal lobe [171]. Similar increases were reported using [³H] D-aspartate [184] but another study failed to replicate the finding in putamen [180]. Studies using [³H]TCP (which, similar to MK801, binds to the phencyclidine site on the NMDA receptor complex) have also resulted in conflicting observations with either no change [185] or an increase in binding in orbitofrontal cortex being reported [186].

More recent studies have examined the expression of NMDA receptor subunits in schizophrenic brain and have reported several region-specific results. There are reports of increased temporal and PFC expression of NR1 [187–189]. However, an older study [190] had found no major changes in any of the NMDA receptor subunits in PFC with the exception of a higher ratio of NR2D to the other NR2 subunits. In the thalamus, a significant reduction in NR1, NR2A, NR2B, and NR2CR subunits have been reported [178, 191, 192]. Other reports demonstrated a downregulation of

NR1 in the superior temporal gyrus, hippocampus [182, 193], and thalamus [178] and upregulation of NR2B subunit in the superior temporal cortex [188]. A recent study also reported a decrease in NR1 subunit expression in substantia nigra [167].

Studies examining the expression of the family of metabotropic glutamate receptors have only recently begun, and although there are only a few published studies in this area [194, 195], this is likely to be an active field of research in the future. So far, an increase in mGluR5 in orbitofrontal cortex has been reported [194].

In addition to glutamate receptors, Coyle and coinvestigators [196] have reported postmortem abnormalities in the expression of the neuropeptide *N*-acetylaspartyl glutamate (NAAG), which is considered an endogenous ligand for some subtypes of glutamate receptors. This reported increase in the levels of NAAG as well as a decrease in its catabolic enzyme NAALADase in the PFC and hippocampus may reflect alteration in glutamate neurotransmission in schizophrenia [197]. Abnormalities of other indices of glutamate transmission, such as a decrease in vesicular transporter 1, have also been reported in the medial temporal cortex of schizophrenic brains [198]. In the thalamus, an increase in excitatory amino acid transporters EAAT1 and EAAT2 and vesicular glutamate transporter VGLUT2 and in the NMDA receptor-associated proteins NF-L, PSD95, and SAP102 has been reported [199–201].

Another approach has been to investigate mechanisms that indirectly affect the NMDA receptor function, including those that involve the glycine modulatory site on this receptor. Preliminary evidence suggests that the levels of endogenous agonists (glycine and D-serine) and antagonist (kynurenic acid) for this site may have been altered in schizophrenia. This includes reports of decreased serum levels of D-serine [202] and glycine [203] and increased CSF levels of kynurenic acid [204–206] in patients with schizophrenia.

Pathological evidence of abnormalities in structural organization of frontal cortices in schizophrenia may be an indirect indicator of disruption in the main mode of chemical communication (glutamatergic transmission) in this region. Accordingly, abnormalities in a range of structural parameters, including gray matter volume [207–212], cortical thickness [213], cortical gyrification [214, 215], hippocampal shape [216, 217], neocortical and hippocampal pyramidal cell size [218–226], and dendritic spine number and arborization [227–230], have been reported in schizophrenia. Furthermore, several groups have reported the presence of aberrantly located or clustered neurons in entorhinal cortex [231–233] and neocortical white matter [234–239] of schizophrenic brains, findings that fit the idea of an early neurodevelopmental problem in cortical areas. While these pathological abnormalities should be interpreted with caution because of the relatively small effect of sizes and inconsistencies in reported changes in any single parameter, together they further support the involvement of the cortical and thalamic glutamatergic system in the pathophysiology of schizophrenia.

8.3.3 Imaging Evidence

Unlike the monoamine systems, selective glutamate receptor ligands for clinical imaging studies have not been fully developed for routine use in healthy and patient volunteers [240]. As a result, most of the imaging evidence regarding a link between glutamate and schizophrenia is indirect. This includes evidence of abnormal functional

connectivity in frontotemporal cortices during working memory tasks in schizophrenic patients [241–247]. In addition, an impaired recruitment of hippocampus during a memory recollection task [248] and an abnormal frontotemporal interaction during a semantic processing task [249] have been reported in schizophrenic patients. Offering another indirect line of evidence, Kegeles et al. [250] used SPECT and proton magnetic resonance spectroscopy (MRS) to study, in normal subjects, the effect of an experimental state of NMDA receptor hypofunction (induced by ketamine) on amphetamine-induced striatal dopamine release, a measure previously reported to be enhanced in schizophrenia [60] (see Section 8.2). They found an increase in raclopride displacement, interpreted as enhanced dopamine release, by an NMDA receptor antagonist. This finding is similar to observations in schizophrenic patients and suggests that NMDA receptor hypofunction may explain the enhanced dopaminergic response to amphetamine challenge in schizophrenia.

8.3.4 Genetic Evidence

While there is limited evidence for the direct involvement of a glutamate receptor gene in schizophrenia (see [251, 252] for a review), recent genetic studies offer a novel insight for possible pathophysiological processes that converge on glutamate-related targets (see below). A linkage study with a genetically isolated African population suggested that a NR1 subunit polymorphism might be associated with predisposition to develop schizophrenia [253]. Other studies, however, have so far reported lack of association with polymorphisms for genes encoding for NR1, NR2B, GluR5, mGluR7, and mGluR8 [254–257]. So far, the most interesting link to a glutamate receptor gene has been suggested for mGluR3 (*GRM3*) in three independent association studies [258–260]. Egan and coinvestigators [258] have shown that a variation in this gene can affect performance on working memory and attention tasks and on functional magnetic resonance imaging (fMRI) activation of dorsolateral PFC and hippocampus in both schizophrenic patients and normal controls. Interestingly, the agonists of type II mGlu receptors (including mGluR2 and mGluR3) can reverse the adverse behavioral, cognitive, and electrophysiological effects of NMDA receptor antagonists [261, 262]. However, further investigation on the role of this candidate gene is required since there are reports of unaltered *GRM3* mRNA level in PFC and thalamus of schizophrenic patients [194, 195, 263].

Regardless of direct involvement of glutamate receptor genes, most of the candidate genes that have emerged from recent association and linkage studies are functionally linked to glutamatergic transmission (see [166, 264] for a review). These genes include, but are not limited to, neurogelin 1 (*NRG1*), regulator of G-protein signaling 4 (*RGS4*), *G72*, dysbindin (*DTNBP1*), *PPP3CC*, disrupted-in-schizophrenia 1 (*DISC1*), and proline dehydrogenase (*PRODH2*). All of these candidate genes may interact with the glutamatergic transmission and function at various postsynaptic levels. For example, *NRG1* regulates the expression of glutamate receptor subunits and directly activates ErbB4, a tyrosine kinase that regulates the kinetic properties of NMDA receptor [265, 266]. *RGS4* can inhibit the mGlu5 receptor transmission through negative regulation of G-protein signaling [267–269]. *G72* interacts with the enzyme D-amino acid oxidase (DAAO) (see above) that reduces the synaptic availability of D-serine through oxidizing it [270, 271]. Dysbindin recruits nitric oxide synthase (NOS), which in turn affects NMDA receptor activity [272].

PPP3CC encodes a subunit of calcineurin that is considered essential for some types of NMDA receptor-mediated plasticity [273]. Implication of these genes has prompted the suggestion that they may functionally converge at the level of microcircuit information processing, particularly involving NMDA receptor pathways [166, 264].

8.3.5 Pharmacological Evidence

It is plausible that the next generation of antipsychotic therapies may eventually emerge from current research on glutamatergic transmission. In contrast to the dopamine field that was originally derived by the effectiveness of serendipitously discovered drugs, glutamate-based therapeutics would conceptualize the efforts to translate current neuroscientific ideas into clinical treatments. However, the progress in this field has been slowed by the widespread distribution of major glutamate receptors such as NMDA or AMPA receptors and their involvement in key functions such as learning and memory, raising the concerns that drugs that directly target these receptors may be associated with serious side effects. To tackle this caveat, some researchers have attempted to use coagonists of NMDA receptor that bind to its glycine site and increase the frequency of NMDA-gated channel opening [274–278]. Basic research has indicated that the glycine modulatory site on NMDA receptor is not saturated in vivo and thus glycine and related molecules such as D-serine and D-cycloserine may be able to ameliorate the NMDA receptor hypofunction (see [279] for a review). Early clinical trials have indicated that this strategy may alleviate the negative and perhaps cognitive symptoms of schizophrenia [276, 280–285]. However, these agents do not appear to have antipsychotic efficacy and their effectiveness as adjuvant therapy remains to be established [286–289]. Notably, a recent meta-analysis of the randomized controlled trials in schizophrenic subjects showed a moderate amelioration of negative symptoms by glycine and D-serine without any significant effect on other symptoms [290]. Meanwhile, some have suggested that a positive modulation of the glycine site may best be achieved through manipulating the molecular pathways that regulate the turnover of its endogenous ligands. For example, it is known that the availability of glycine is dependent on the activity of glycine transporter 1 (GlyT1) [291, 292], while the availability of D-serine is determined by the activity of serine racemase and the degrading enzyme DAO [293]. Thus, efforts are underway to develop inhibitors of GlyT1 and serine racemase in order to increase the in vivo availability of glycine and D-serine, respectively [278, 279, 294, 295]. Another line of potential therapies that is currently under investigation is based on fine tuning glutamate transmission through metabotropic glutamate receptors [296, 297]. In this regard, one promising strategy may be based on functional potentiation of postsynaptic mGlu5 receptors that show synergistic interactions with NMDA receptors in regulation of behavior and cognition in animal studies [298–301]. Findings from these studies predict that activation of mGlu5 receptors may ameliorate the presumed state of NMDA receptor deficiency in schizophrenia. However, the rapid desensitization rate of mGlu5 receptors makes it unlikely that direct agonists of these receptors may have persistent therapeutic effectiveness. Instead, recently developed positive allosteric modulators of mGlu5 receptor function may offer a more efficacious strategy [302–304], a promise backed by recent reports in experimental models [305, 306, 306a]. Alternatively, preclinical

studies have shown that the agonists of group II mGlu receptors may inhibit the adverse effects of NMDA receptor antagonists on behavior, working memory, and PFC function [261, 262, 307], suggesting the therapeutic potential of this strategy for schizophrenia. A key finding was that NMDA receptor antagonists lead to an excessive increase in PFC glutamatergic transmission through non-NMDA receptors [308], leading to the assumption that agents such as activators of presynaptic group II mGlu autoreceptors that can block this aberrant activity may prove beneficial to relieve the presumed states of cortical malfunction. Accordingly, a recent clinical study in healthy human subjects treated with ketamine offered the first proof of concept that activation of group II mGluRs may ameliorate cognitive dysfunction in the context of NMDA hypofunction [309]. The same principle, normalizing an inappropriate pattern of cortical activity, may underlie the reported effectiveness of lamotrigine, an anticonvulsant agent with cation channel blockade properties, in ketamine-treated subjects [310, 311]. Correspondingly, preliminary reports of the advantageous effects of lamotrigine adjunctive therapy in schizophrenia have been published [312–314].

Another approach has been to develop agents that reduce the rate of desensitization and therefore potentiate the function of AMPA glutamate receptors as “cognitive enhancers” since these agents can improve learning and memory in animal models [315–318]. Preliminary results with this class of agents, called “AMPAkines,” suggest that boosting glutamatergic transmission may indeed be beneficial for the core cognitive deficits in schizophrenia [319]. However, these drugs may be in particular worthy as adjuvant to current therapies [319, 320], rather than for single-agent therapy [321].

Given that our approach to treating schizophrenia has not fundamentally changed for 50 years and the acute need to develop more efficacious drugs for treatment of nonpsychotic symptoms of schizophrenia, in particular the enduring cognitive deficits of the disease, there has been a surge of interest in developing and testing “novel” glutamatergic drugs in animal models and in human subjects. Hence, the next decade promises to be an exciting time for the field because, for the first time, drugs that are designed based on pathophysiological mechanisms and are not merely prototypes of existing neuroleptics may be developed.

8.4 CONSOLIDATING GLUTAMATE AND DOPAMINE HYPOTHESES OF SCHIZOPHRENIA

Given the dominance of the dopamine hypothesis, the original attempts to consolidate these theories focused on proving that NMDA receptor deficiency is associated with dopamine hyperactivity. Earlier release studies examining dopamine turnover or measuring the uptake of labeled dopamine showed that NMDA receptor antagonists increase the release of striatal dopamine [322–324]. This finding contributed to the first version of an integrated dopamine–glutamate theory, suggesting that NMDA receptor hypofunction may represent a paradigm that simulates the presumed dopamine hyperfunction in schizophrenia [154, 325, 326]. This interpretation, however, could not account for the fact that NMDA receptor antagonists induce a far wider range of schizophrenia-like symptoms than the dopaminergic models. Thus, the theory was modified to distinguish a role for cortical

versus subcortical glutamate–dopamine interactions for induction of negative and cognitive versus positive symptoms [50, 114, 160]. However, subsequent work using microdialysis or imaging methodologies showed that behaviorally relevant doses of NMDA antagonists do not increase dopamine release in rodents, primates, or humans [327–330]. Furthermore, behavioral studies demonstrated that the dopamine system is neither necessary [331] nor sufficient [327] for maintenance of the aberrant behavioral effects of the NMDA receptor antagonists. This and the abundance of postmortem and genetic findings supporting a role for the glutamate system in the etiology and pathophysiology of schizophrenia have led to the proposal that the primary abnormalities in schizophrenia may involve the synaptic signaling machinery in cortical regions [2, 92, 332] and that a dopaminergic abnormality may be a consequence of cortical dysregulation of dopamine neurons. Interestingly, contrary to years of assertion that the PFC stimulates dopamine neuronal activity [333, 334], recent anatomical studies have demonstrated that cortical projections do not synapse directly onto mesostriatal dopamine neurons [335]. Furthermore, stimulation of PFC neurons at physiological frequencies actually decreases dopamine release in the ventral striatum [336]. This suggests that the PFC exerts an inhibitory influence over subcortical dopamine presumably through indirect activation of GABA neurons [336, 337]. Thus, reduced glutamatergic function in the PFC may remove this inhibitory influence and lead to an abnormally overactive subcortical dopamine system in schizophrenia. Recent electrophysiological studies recording from PFC neurons in behaving rodents, in fact, show that a state of NMDA deficiency can lead to reduced burst activity of cortical neurons [338].

Given the lack of direct evidence for a dopaminergic abnormality in schizophrenia, an alternative hypothesis has been that antipsychotic drugs, which are D_2 receptor antagonists, work by modifying the function of cortical (glutamatergic) neurons [166]. A substantial body of evidence demonstrates that dopamine modulates cortical and subcortical glutamatergic transmission [339–345]. Notably, electrophysiological studies have revealed a delicate modulatory effect for dopamine on the electrical conductance of cortical excitatory neurons, that is, neither excitatory nor inhibitory, but rather is a gating effect that depends on the activity state of target neurons [341, 344, 345]. Furthermore, D_2 receptors may regulate the temporal organization of electrical activity in PFC [343]. The D_2 receptors also inhibit the release of glutamate [346, 347], suggesting that blockade of D_2 receptors by antipsychotic drugs can overcome a putative state of glutamate deficiency. In support of this mechanism, electrophysiological studies have shown that antipsychotic agents, particularly clozapine, exert positive modulatory effects on the NMDA receptor function in PFC and may attenuate the blockade of these receptors by phencyclidine [348–351]. Interestingly, a recent study in behaving animals suggests that clozapine can reverse the disruptive effects of NMDA receptor blockers on cortical firing in correlation with behavior. This reversal may be a result of fine-tuning of cortical activity as clozapine increased the activity of the neurons with low baseline firing rates and decreased the activity of neurons with higher firing rates [351a].

Another hypothesis has been that dopamine and glutamate interactions may occur at the postsynaptic level and through the intricate postsynaptic intracellular mechanisms known to mediate crosstalks between these transmitter systems [352, 353]. Taken together, these modified scenarios would explain the lack of strong

pathological and genetic evidence for the involvement of dopamine in the pathophysiology of schizophrenia despite the clinical effectiveness of dopamine-based drugs. Instead, they emphasize the importance of viewing schizophrenia as a constellation of molecular and cellular processes that may functionally converge at the circuitry level, most probably downstream from corticolimbic NMDA receptors. One implication of these models is that polytransmitter theories, incorporating interactions between dopamine, glutamate, and other major neurotransmitter systems, have replaced the existing monotransmitter theories [342]. An important example in this case is the PFC GABAergic system which has been strongly linked to schizophrenia (see [354, 355] for a review). It has been hypothesized that disruptions in GABA-mediated inhibitory tone of cortical interneurons may contribute to decrease the signal-to-noise ratio in cortical circuitry in schizophrenia [341, 342], paving the way for the sort of functional degradation that occurs in the NMDA antagonist model [338, 356].

Rapid progress in the fields of imaging, psychiatric genetics, and postmortem molecular analysis of brain tissues is likely to help continue the evolution of dopamine and glutamate hypotheses of schizophrenia. While one hopes that for a devastating disease like schizophrenia hypotheses will soon be replaced by concrete data and effective cures, the concerted efforts of researchers in conceptualizing theoretical bases of schizophrenia has been, and will continue to be, instrumental in developing a better understanding of the mechanisms underlying schizophrenic disorders and efforts to develop a new generation of more effective treatments.

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9

MOLECULAR GENETICS OF SCHIZOPHRENIA

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9.1	Genetic Epidemiology	321
9.1.1	Defining Phenotype for Genetic Research	322
9.1.2	Are There Clues for Genetics from Epidemiology, Pathophysiology, and Neurobiology?	323
9.2	Molecular Genetic Studies	323
9.2.1	Linkage Studies	323
9.2.2	Positional Candidate Genetics	325
9.3	Candidate Genes	325
9.3.1	Dystrobrevin Binding Protein 1 (DTNBP1)	325
9.3.2	Neuregulin 1	326
9.3.3	D-Amino Acid Oxidase (DAO) and D-Amino Acid Oxidase Activator (DAOA)	327
9.3.4	Regulator of G-Protein Signaling 4	328
9.3.5	Others	328
9.4	Chromosomal Abnormalities	328
9.4.1	Catechol-O-Methyltransferase (COMT)	328
9.4.2	PRODH	329
9.4.3	ZDHHC8	330
9.4.4	DISC1	330
9.5	Functional Candidate Genes	331
9.6	Functional Implications of Susceptibility Genes	332
9.7	Conclusions	333
	References	333

9.1 GENETIC EPIDEMIOLOGY

The results of numerous family, twin, and adoption studies show conclusively that risk of schizophrenia is increased among the relatives of affected individuals and that

is the result largely of genes rather than shared environment [1–3]. In the children and siblings of individuals with schizophrenia, the increase in risk is around 10-fold and somewhat less than this in parents. The latter finding is probably explained by a reduction in the reproductive opportunities, drive, and possibly fertility of affected individuals. Five recent systematically ascertained studies report monozygotic (MZ) concordance estimated at 41–65% compared with dizygotic (DZ) concordance of 0–28% and an estimate of broad heritability of 85% [4]. The heritability of schizophrenia is one of the highest for any complex genetic disorder. To place it in perspective, it is similar to that of type 1 diabetes (72–88% [5, 6]) but greater than breast cancer (30% [7]), coronary heart disease in males (57% [8]), and type II diabetes (26% [9]).

While the twin and adoption literature leaves little doubt that genes are important, they also point to the importance of environmental factors since the concordance for schizophrenia in MZ twins is typically around 50% and heritability estimates are less than 100%. Moreover, we should also note that risks resulting from gene–environment interactions tend to be attributed to genes in most genetic epidemiological studies.

Genetic epidemiology also tells us that, like other common disorders, schizophrenia has a mode of transmission that is complex and includes the multiple-susceptibility-loci model [10, 11]. However, the number of loci, the risk conferred by each, and the degree of interaction between them remain unknown. Risch [12] has calculated that the data are incompatible with the existence of a single locus conferring a sibling relative risk (λ_s) of more than 3 and, unless extreme epistasis (gene–gene interaction) exists, models with two or three loci of $\lambda_s \leq 2$ are more plausible. It should be emphasized that these calculations are based upon the assumption of homogeneity and refer to populationwide λ_s . It is quite possible that alleles of larger effect are operating in some groups of patients, for example families with a high density of illness. However, high-density families are expected to occur by chance even under polygenic inheritance, and their existence does not prove the existence of disease alleles of large effect [11].

9.1.1 Defining Phenotype for Genetic Research

Schizophrenia displays considerable heterogeneity of symptoms, course, and outcome (see Chapter 7). It is possible that this reflects etiological heterogeneity, although if etiologically distinct subgroups do exist, we cannot yet identify them. In spite of this, structured and semistructured interviews together with explicit operational diagnostic criteria permit reliable diagnosis of a syndrome with high heritability. It should then in principle be possible to subject schizophrenia to molecular genetic analysis.

One way of refining the phenotype for genetic studies of complex traits is to define intermediate phenotypes or endophenotypes, that is, traits that are intermediate between susceptibility genes and the clinical phenotype. A number of endophenotypes for schizophrenia have been proposed based upon electrophysiology, pharmacology, psychology, or neuroimaging. Such traits are likely to be essential for understanding how variation in a proven susceptibility gene leads to the clinical phenotype. However, the use of endophenotypes for the identification of disease genes requires that the measures in question are trait, rather than state, variables and compelling

evidence from genetic epidemiology that they reflect genetic vulnerability to that disease rather than environmental factors. Promising data are accumulating for some potential endophenotypes for schizophrenia [13], but, in most cases, uncertainties remain.

The situation is further complicated by the fact that we are unable to define the limits of the clinical phenotype to which genetic liability can lead. It clearly extends beyond the core diagnosis of schizophrenia to include a spectrum of disorders, including schizoaffective disorder and schizotypal personality disorder [14, 15]. However, the limits of this spectrum and its relationship to other psychotic disorders, especially bipolar disorder, remain uncertain [16, 17]. Based upon the way the field is progressing, it seems likely that one of the earliest benefits of the identification of susceptibility genes is that the validity of current nosological categories can be further explored. Knowing the genes involved should allow us to dissect the current concept of schizophrenia and help us to understand its relationship to other diagnostic groups.

9.1.2 Are There Clues for Genetics from Epidemiology, Pathophysiology, and Neurobiology?

Epidemiological, pharmacological, and neurobiological studies have made some progress in our general understanding of schizophrenia (see other chapters in this section). The more specific hypotheses based upon abnormalities in neurotransmission, especially dopaminergic and glutaminergic, are very possibly relevant to some of the overt clinical manifestations of the disorder, but with few exceptions, molecular genetic studies predicated on these hypotheses have met with disappointing results. Moreover, many of the leading hypotheses involve rather vague concepts such as neurodevelopment, synaptic dysfunction, and aberrant neuronal connectivity. These concepts are so broad that it is difficult to use them to confidently implicate specific pathophysiological processes or to specify compelling candidate genes. These problems with hypothesis-based approaches have encouraged a number of groups to apply so-called positional genetic approaches for the simple reason that these do not depend upon knowledge of disease pathophysiology.

9.2 MOLECULAR GENETIC STUDIES

9.2.1 Linkage Studies

In contrast with several other common disorders like breast cancer, Alzheimer's disease, Parkinson's disease, and epilepsy, no genetically simple subtypes of schizophrenia have yet been discovered. The results of linkage studies in schizophrenia have often seemed to be disappointing, with positive studies usually falling short of stringent "genomewide" levels of significance and abundant failures to replicate. This is probably attributable to a combination of small genetic effects, inadequate sample sizes, and heterogeneity of causative loci between and within populations [18]. However, as more than 20 genomewide studies have been reported, and sample sizes increased, some consistent patterns have emerged. Linkages that reached genomewide significance on their own according to the criteria set forth by Lander

and Kruglyak [19] or those that have received strong support from more than one sample are shown in Figure 9.1.

Recently, two meta-analyses of schizophrenia linkage have been reported. Each used different methods and obtained overlapping but somewhat different results. The study of Badner and Gershon [20] supported the existence of susceptibility genes on chromosomes 8p, 13q, and 22q, while that of Lewis et al. [21] most strongly favored 2q. The latter study also found that the number of loci meeting the aggregate criteria for significance was much greater than expected by chance ($p < 0.001$), with evidence for susceptibility genes on chromosomes 5q, 3p, 11q, 6p, 1q, 22q, 8p, 20q, and 14p.

The linkage data therefore support the predictions made by Risch [12] on the basis of genetic epidemiological findings; that is, the evidence is consistent with the existence of multiple susceptibility alleles of moderate effect. Nevertheless, it is also possible that loci of larger effect exist in specific samples of large multiply affected families. Of course, the proof that a positive linkage is correct comes when the disease gene has been identified. Happily, recent years have finally seen a number of breakthroughs in post-linkage positional cloning.

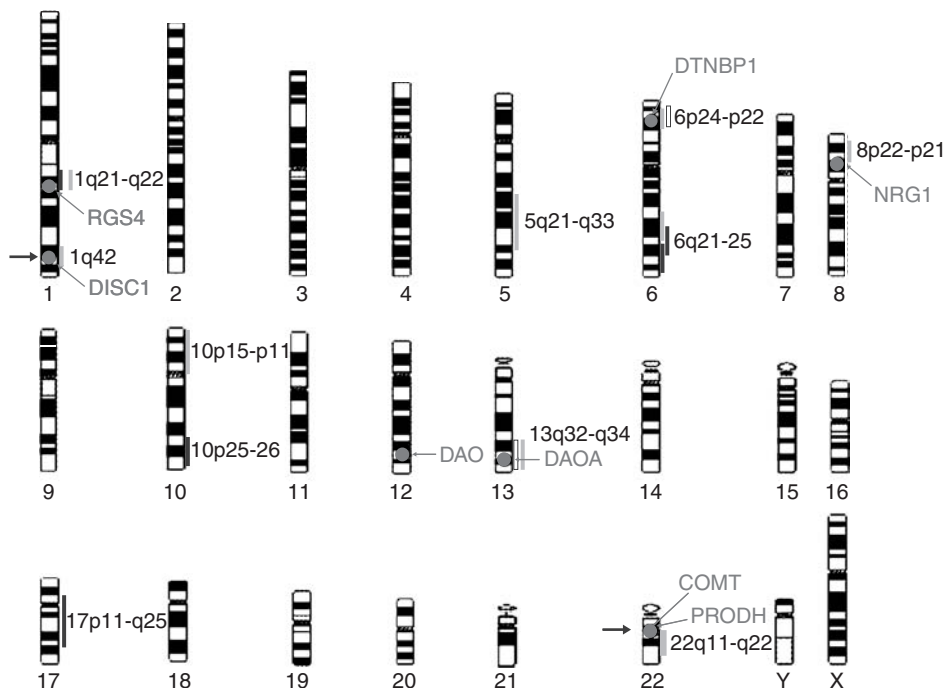


Figure 9.1 Ideogram showing chromosomal regions of linkage to schizophrenia and candidate genes that may be associated. Vertical bars denote regions where evidence for linkage has been found in more than one study (green) and where linkage has reached genomewide significance (red) according to the criteria of Lander and Kruglyak [19]. Arrows (red) identify regions where chromosomal abnormalities are involved with schizophrenia. The chromosomal positions of the major candidate genes discussed in this chapter are also displayed. (See color insert.)

9.2.2 Positional Candidate Genetics

Previously, the economics and practicalities of hunting a susceptibility gene within a linked chromosomal region dictated that one should have virtually definitive evidence that the linkage was a true positive. With recent improvements in our knowledge of genome anatomy and in genome analysis technology, the task of positional cloning has been transformed and favors bolder endeavors. These considerations, together with the convergence of some positive linkage findings, have led to a number of detailed mapping studies of linked regions which have in turn implicated specific genes. However, the quality of the data has been variable and a number of putative susceptibility genes have yet to be clearly replicated. Here, we focus on the genes where, at the time of writing, there are published follow-up studies or where we are aware of data that allow judgment about whether the gene is likely to be a true positive. In making these judgments, we have primarily been influenced by the strength of the genetic evidence for association to the clinical phenotype. We have given no weight to claims that a SNP (single-nucleotide polymorphism) is functional or that the gene has plausibility by virtue of patterns of expression or participation in relevant pathophysiological pathways. These are key properties of true susceptibility variants, but the number of SNPs in genes that meet the above criteria is so vast that such evidence cannot be used to prop up weak genetic findings. We have also given little weight to associations with putative endophenotypes, be they based on electrophysiology, pharmacology, psychology, or neuroimaging or performed in humans or modeled in animals. Such studies are likely to be essential for understanding how variation in a proven susceptibility gene leads to the clinical phenotype. However, for the *de novo* identification of disease genes, unless it is known with a very high degree of confidence that an endophenotype in question does actually index genetic risk for disorder, a considerable amount of uncertainty inevitably must accompany its use.

9.3 CANDIDATE GENES

9.3.1 Dystrobrevin Binding Protein 1 (DTNBP1)

Chromosome 6p22.3 is one of the most consistently reported linkage regions for schizophrenia (Fig. 9.1). After detailed studies designed to follow up their original findings of linkage in Irish pedigrees, Straub and colleagues [22] reported evidence for genetic association between schizophrenia and markers in the *DTNBP1* or dysbindin gene which maps to this region. Significant associations to markers and haplotypes have subsequently been found by several other research groups in independent populations of schizophrenics (e.g., [23–29]) which have included samples that do not show significant linkage to the 6p region. Although not unexpectedly there have also been some samples in which association has not been found [9, 26, 30], the evidence now strongly favors *DTNBP1* as a susceptibility gene for schizophrenia and possibly a more general psychotic phenotype [32]. However, the associated haplotypes have differed between studies, suggesting possible allelic heterogeneity or, alternatively, population differences in the linkage disequilibrium structure across the gene [23].

As yet, no causative variant has been identified, and the absence of associated nonsynonymous alleles after systematic mutation detection of the exons [25] suggests that disease susceptibility depends upon variation affecting messenger RNA

(mRNA) expression or processing. The latter possibility is *directly* supported by evidence for as-yet-unknown cis-acting alleles affecting *DTNBP1* expression in the human brain [33] that may reside upon haplotypes reported to be overrepresented in schizophrenics [37] and also by two recent studies showing reduced levels of expression of the mRNA [34] and protein [35] in post-mortem brain samples from patients with schizophrenia. This hypothesis needs to be tested by studying the relationship between the various associated haplotypes and RNA abundance, but identification of the risk variant, or variants, will require high-resolution work, including the identification and analysis of all polymorphisms across *DTNBP1*, possibly in multiple populations.

Dysbindin binds both α - and β -dystrobrevin, which are components of the dystrophin glycoprotein complex. The dystrophin complex is found in the sarcolemma of muscle but is also located in postsynaptic densities in a number of brain areas, particularly mossy fiber synaptic terminals in the cerebellum and hippocampus. Its location initially suggested that variation in *DTNBP1* might confer risk of schizophrenia by mediating effects on *postsynaptic* structure and function [22]. However, dysbindin has been shown to be part of a complex that influences the activity of presynaptic intracellular vesicles [36]. Talbot and colleagues [35] have recently shown that the presynaptic dystrobrevin-independent fraction of dysbindin is reduced in schizophrenic brain within certain intrinsic glutamatergic neurons of the hippocampus. Talbot and colleagues also observed that in schizophrenic brain reduced dysbindin expression was associated with increased expression of the vesicular glutamate transporter type 1. Taken together with the further observation that reduction in *DTNBP1* expression [27] is associated with reduced glutamate release *in vitro*, the findings so far are compatible with the hypothesis that *DTNBP1* might confer risk by altering presynaptic glutamate function.

9.3.2 Neuregulin 1

Neuregulin (*NRG1*) was first implicated in schizophrenia in an Icelandic sample [38]. Association analysis across a schizophrenia linkage region on 8p21–22 revealed highly significant evidence for association between schizophrenia and a multimarker haplotype at the 5' end of *NRG1*. Strong evidence for association with the same at-risk haplotype was subsequently found in a large sample from Scotland [39] and weakly replicated in a U.K. sample [40]. Further positive findings have emerged from Irish [41], Bulgarian (Kirov et al., unpublished), Chinese [30, 42–44], and South African [30] samples that largely implicate the 5' region of the gene. However, some negative findings have also been reported [30, 45–47], notably in Irish, Japanese, U.S. and South African populations. Only the two deCODE studies and our own U.K. study have implicated the specific Icelandic haplotype, perhaps reflecting differences in the LD (linkage disequilibrium) structure across *NRG1* in European and Asian samples (e.g., [44]) and emphasizing the fact that the associated variants, even if true positives, are not the causative ones.

Despite detailed resequencing [38], it has not yet proven possible to identify specific susceptibility variants, but the Icelandic haplotype points to the 5' end of the gene, once again suggesting that altered expression or perhaps mRNA splicing might be involved. It is even formally possible at this stage that *NRG1* is not itself the susceptibility gene, as intron 1 contains another expressed sequence [41] whose

function is unknown. However, insofar as it is possible to model schizophrenia in animals, behavioral analyses of *NRG1* hypomorphic mice support the view that the association is related to altered *NRG1* function or expression [48]. More direct evidence suggesting that altered expression, and in particular altered expression of the ratios of different *NRG1* transcripts, might be involved in the pathogenesis of schizophrenia is also beginning to accumulate [49].

Just as for *DTNBP1*, the pathological pathways by which altered *NRG1* function might lead to schizophrenia are unclear. *NRG1* encodes many mRNA species, which in turn translate into numerous proteins with multifarious functions. At the time it was implicated as a susceptibility gene for schizophrenia, it was thought to encode around 15 proteins with a diverse range of functions in the brain, including cell–cell signaling, ErbB receptor interactions, axon guidance, synaptogenesis, glial differentiation, myelination, and glutamatergic neurotransmission [50]. Any, or perhaps a combination of several of these, could be involved. Moreover, to further complicate matters, a number of novel exons that encode novel *NRG1* isoforms whose functions are currently unknown have recently been identified [51].

9.3.3 D-Amino Acid Oxidase (*DAO*) and D-Amino Acid Oxidase Activator (*DAOA*)

Chumakov and colleagues [52] undertook association mapping in the schizophrenia linkage region on chromosome 13q22–34. They found evidence for association to markers around two novel genes they termed *G72* and *G30* in French Canadian and Russian populations. *G72* and *G30* are overlapping but transcribed in opposite directions. Little is known of *G30* other than that it is transcribed in the brain. *G72* is a primate-specific gene expressed in the caudate and amygdala. Using yeast two-hybrid analysis, Chumakov and colleagues reported evidence for physical interaction between *G72* and *DAO*. *DAO* is expressed in human brain where it oxidizes D-serine, a potent activator of NMDA glutamate receptors. Coincubation of *G72* and *DAO* in vitro revealed a functional interaction between the two, with *G72* enhancing the activity of *DAO*, and consequently, *G72* has now been named *DAOA*. In the same study, *DAO* polymorphisms were shown to be associated with schizophrenia in the Canadian sample only, and analysis of *DAOA* and *DAO* variants revealed modest evidence for a statistical interaction between the loci and disease risk. Given the three levels of interaction, the authors concluded that both genes influence risk of schizophrenia through a similar pathway and that this effect is likely to be mediated through altered NMDA (*N*-methyl-D-aspartate) receptor function.

Associations between *DAOA* and schizophrenia have subsequently been reported in samples from Germany [53], China [54, 55], and both the United States and South Africa [30] and in Askenazi Jews [56] and a small sample of very early onset psychosis subjects from the United States [57]. As before, and conceivably for similar reasons, there is no consensus concerning the specific risk alleles or haplotypes across studies. At present, the published genetic evidence in support of both of these genes is weaker than for *DTNBP1* and *NRG1* and stronger for *DAOA* than *DAO*; indeed, so far, only a German group [53] has reported (weak) evidence in favor of *DAO*. Nevertheless further research is clearly required given reports of association of *DAOA* with bipolar disorder and psychosis [57, 58] suggesting that the gene might be a susceptibility locus for some aspect of psychopathology that is present in both schizophrenia and bipolar disorder.

9.3.4 Regulator of G-Protein Signaling 4

RGS4 maps to a putative linkage region on chromosome 1q22, but it was targeted for genetic analysis [59] following a microarray-based gene expression study in which decreased *RGS4* expression was found in schizophrenic postmortem brain [60]. Evidence for association between schizophrenia and haplotypes at the 5' end of the gene was found in two samples from the United States, and while not providing significant evidence alone, inclusion of a sample from India added to the overall level of support [59]. Analysis of a Brazilian sample has not provided support [61], but positive findings with both single markers and haplotypes have been reported in samples from the United Kingdom and Ireland [62–64]. The level of support for each has been modest and the pattern of association different between samples. Currently, our view is that the evidence for *RGS4* is interesting but far from convincing, and the results of a meta-analysis being coordinated by the original group to which the present authors have contributed are now keenly awaited.

In terms of possible mechanisms of action, *RGS4* is a negative regulator of G-protein-coupled receptors. The relationship between RGS molecules and receptor function is a promiscuous one, but of possible interest to schizophrenia is the evidence that *RGS4* modulates activity at certain serotonergic [65] and metabotropic glutamatergic receptors [66], while its own expression is modulated by dopaminergic transmission [67]. Moreover, *RGS4* interacts with *ErbB3* [68], which may be of relevance as *ErbB3* is a *neuregulin 1* receptor whose expression is downregulated in schizophrenic brains [50]. Interestingly, variation between *RGS4* has also recently been associated with reduction in dorsolateral prefrontal cortex gray matter volume [69], a finding of possible relevance given that reduction in prefrontal gray matter is well documented in schizophrenics (e.g., [70]).

9.3.5 Others

Association has been claimed between schizophrenia and *CAPON* (C-terminal PDZ domain ligand of neuronal nitric oxide synthase) [71], *PPP3CC* (protein phosphatase 3, catalytic subunit) [72], *TRAR4* (trace amine receptor 4) [73], and enthoprotin [74], which map respectively to putative linkage regions on 1q22, 8p21.3, 6q23.2, and 5q33. As discussed in the original articles, each of these genes can be plausibly related to candidate pathophysiologies of schizophrenia, but at the time of writing, we are not aware of any robust replication data to support these hypotheses, although *CAPON* has received some support in a Chinese sample [75].

9.4 CHROMOSOMAL ABNORMALITIES

9.4.1 Catechol-*O*-Methyltransferase (*COMT*)

There have been numerous reports of associations between schizophrenia and chromosomal abnormalities [76], but with two exceptions none provides convincing evidence for the location of a susceptibility gene. Several studies have shown that adults with 22q11 deletions have a high risk for schizophrenia [77–79], with the largest study of adult patients to date ($n = 50$) estimating this at 24% [79]. The deletion cannot account for a high proportion of schizophrenic cases [80], but reports

of linkage to 22q11 [20, 21] suggest that variants in genes mapping to this region might contribute to more typical cases. Current candidates include *COMT*, proline dehydrogenase (*PRODH*), and zinc finger- and DHHC domain-containing protein 8 (*ZDHHC8*).

COMT has been intensively studied because of its key role in dopamine catabolism. Most studies have focused upon a valine-to-methionine change at codon 158 of the brain predominant membrane-bound form of COMT (MB-COMT) and codon 108 of the soluble form (S-COMT). The valine allele confers higher activity and thermal stability to COMT [81] and has been fairly consistently associated with reduced performance in tests of frontal lobe function [82, 83]. The results in schizophrenia have been mixed, with recent meta-analyses [84] reporting no overall evidence for association.

Since the preparation of the meta-analysis, an Israeli study of over 700 cases reported strong evidence for association between haplotypes, including the *val158* allele and two flanking, non coding SNPs [85]. As in an earlier study [86] the evidence from haplotypes was stronger than for the valine allele alone, suggesting that *COMT* may well be a susceptibility gene for schizophrenia but that the effect is not attributable to the *val/met* variant. We have been unable to replicate association with any of the SNPs or haplotypes, including the *val/met* polymorphism in a study of more than 2800 individuals, including almost 1200 schizophrenics [87], but two other groups [81, 88] have recently reported rather different haplotype associations at *COMT* in Irish and U.S. samples, respectively. As for the Israeli study, haplotypes carrying the *val158* allele exhibited stronger evidence for association than did that allele alone, while in the second study, the strongest findings included markers spanning the 3' end of the armadillo repeat deleted in the velocardiofacial syndrome gene (*ARVCF*). The latter has also been implicated in an earlier study [86] and its transcribed genomic sequence overlaps with *COMT* [89]. While the picture is confused, we consider that the evidence does not support a role for *val/met 158* in susceptibility to schizophrenia, although a small effect cannot be excluded, nor can a role in phenotype modification. However, it remains a strong possibility that variation elsewhere in *COMT*, or *ARVCF*, confers susceptibility.

9.4.2 *PRODH*

PRODH is another functional candidate gene given that a loss-of-function mutant mouse exhibits behavioral abnormalities in sensorimotor gating that are analogous to those observed in schizophrenics [90] and because proline dehydrogenase influences the availability of glutamate. Evidence in favor of association between SNPs in *PRODH* has been reported [91, 92], but arguing against this, we were unable to replicate either of these findings in large case-control and family-based association samples ([93] and G. Kirov et al., unpublished). Moreover, discrepancies in allele labeling between the two positive studies reduces, though does not abolish, the support provided to the former by the latter. One of the studies [91] also suggested association between a number of *PRODH* missense variants and schizophrenia while a separate study reported both heterozygous deletion of the entire *PRODH* gene in a family that included two schizophrenic subjects and missense variants in 3 of 63 schizophrenic patients studied [94]. However, we [93] and (in a follow-up of their own study [94]) Jacquet and colleagues [95] observed a range of missense mutations to be

equally common in schizophrenic cases and controls, while *PRODH* deletions were reported not to be associated with schizophrenia in a very large sample of Japanese subjects [96]. Interestingly, while the study of Jacquet and colleagues [95] failed to provide evidence for their hypothesis of association between schizophrenia and hyperprolinemia (a consequence of loss-of-function *PRODH* mutations), they did find evidence for association between schizoaffective disorder and *PRODH*. Whether this is a chance finding or a finding of relevance to the discrepancies between existing data is at present unknown.

9.4.3 ZDHHHC8

Finally, there is evidence that a SNP (rs175174) in *ZDHHHC8*, a gene which encodes a putative transmembrane palmitoyl transferase, might directly confer susceptibility to schizophrenia by affecting the splicing of *ZDHHHC8* mRNA [97]. Interestingly, association was only observed in females [97]. The genetic evidence was not strong but gained support from the observation of a similar sexual dimorphism in mice homozygous for a knockout of this gene, with females but not males, displaying the phenotypes modeling aspects of schizophrenia. Unfortunately, of the two published attempts at replication so far, one found the opposite allele to be associated in a Han Chinese sample and no evidence for a gender effect [98] and the other, in a relatively large Japanese case-control study, failed to find any association [99]. Recently in collaboration with colleagues in Bonn [100] we investigated rs175174 in four schizophrenia samples including a Bulgarian proband/parent sample (474 trios) and three case-control panels of European origin (1028 patients, 1253 controls). The results did not support the hypothesis that genetic variation at rs175174 is associated with increased risk for schizophrenia nor did they suggest the presence of gender-specific differences. Overall, the current evidence fails to support the hypothesis that SNP rs175174 in intron 4 of *ZDHHHC8* directly influences susceptibility to schizophrenia. However, although unlikely in our opinion, it remains possible that susceptibility to schizophrenia is conferred by another genetic variant or variants in *ZDHHHC8* that are in LD with rs175174 in the U.S. and South African samples studied by Mukai and colleagues [97] and the Han Chinese sample studied by Chen and colleagues [98] but not in the samples from Bulgaria, Germany, Poland, Sweden, and Japan [99, 100].

9.4.4 DISC1

The other major finding based upon a chromosomal abnormality comes from an extended pedigree in which a balanced chromosomal translocation (1;11)(q42;q14.3) showed strong evidence for linkage to a fairly broad phenotype consisting of schizophrenia, bipolar disorder, and recurrent depression [101]. The translocation was found to disrupt two genes on chromosome 1 which were on this basis called disrupted in schizophrenia 1 and 2 (*DISC1* and *DISC2* [101, 102]). No known genes mapped within the chromosome 11 disruption. *DISC2* contains no open reading frame and may regulate *DISC1* expression via antisense RNA [102]. Interestingly *DISC1* and 2 are located close to the chromosome 1 markers implicated in two Finnish linkage studies [103, 104] (Fig. 9.1). It has been suggested that truncation of *DISC1* in the translocation family might contribute to schizophrenia by affecting

neuronal functions dependent upon intact cytoskeletal regulation such as neuronal migration, neurite architecture, and intracellular transport [105, 106]. While these are interesting hypotheses, it is important to remember that translocations can exert effects on genes other than those directly disrupted. For example, there are several mechanisms by which a translocation can influence the expression of neighboring genes. Thus, in order to unequivocally implicate *DISC1* and/or 2 in the pathogenesis of schizophrenia, it is necessary to identify in another population mutations or polymorphisms that are not in strong LD with neighboring genes but which are associated with schizophrenia. Four published studies have attempted to do this. Negative studies were reported by the Edinburgh group that originally identified *DISC1* and 2 [107] and by a group who focused on the 5' end of the gene in a large Japanese sample [108]. In contrast, positive findings have been reported in a large Finnish study [109] while haplotypic associations were found in U.S. samples with schizophrenia, schizoaffective disorder, and bipolar disorder [110]. At present, the genetic evidence in favor of *DISC1* as a susceptibility gene is gaining momentum but, in our view, is not yet compelling. Interestingly, if *DISC1* is a true susceptibility gene, it appears to confer risk for phenotypes including schizoaffective disorder, bipolar disorder, and major depression as well as schizophrenia [110]. Given the range of phenotypes associated with the translocation in the original study, despite its name, this should not be that surprising.

9.5 FUNCTIONAL CANDIDATE GENES

There is a huge schizophrenia candidate gene literature consisting of negative findings or positive findings that have either not been replicated or have seemed so unconvincing that there have been no attempts to do so. While most of the reported positives are unlikely to stand the test of time, neither can we conclude that any gene has been effectively excluded, given that most have not been studied exhaustively in large samples through a combination of detailed sequencing of exons, introns, and large regions of 5' and 3' flanking sequences combined with exhaustive genotyping. However, meta-analyses do at least suggest that the dopamine receptors *DRD3* [111] and *DRD2* [112] and the serotonergic receptor *HTR2A* [113] might confer risk. The effect sizes, if any, are extremely small [odds ratio (OR) < 1.2] and difficult to confirm, but if the associations are true, the findings provide some support for the view that altered dopaminergic and serotonergic function might be a primary event in schizophrenia susceptibility. If the putative associations are the result of LD between the assayed markers and the true susceptibility variant, it is possible that the latter might contribute somewhat more, depending upon the degree of LD between the two.

Despite the accumulating evidence for abnormalities of glutamatergic neurotransmission in schizophrenia, by and large, analysis of glutamatergic genes as functional candidates for schizophrenia has failed to produce consistent positive findings. However, recently claims have been made that variation in *GRM3* (the gene encoding metabotropic glutamate receptor type 3) might confer risk to schizophrenia. *GRM3* was first implicated in a case-control study of German patients. However, as only one SNP was significantly associated in one out of the three groups of patients studied [114], the authors concluded against a role for this gene in schizophrenia. Fujii and colleagues [115] genotyped six SNPs in a small case-control sample from

Japan. They found significant association with one SNP and a three-marker haplotype containing this SNP. Egan et al. [116] genotyped seven SNPs, including the two implicated in the previous reports, in a family based association study. Weak evidence for overtransmission to cases was observed for one SNP; however, this was not one of the SNPs implicated previously and the result failed to replicate in a second sample. A haplotype including this SNP was also overtransmitted to cases, but this finding has yet to be replicated. The authors presented further data relating the single putatively associated *GRM3* SNP to cognitive, fMRI, MRS, and neurochemical variables. They argued that these data converge on the conclusion that *GRM3* affects prefrontal and hippocampal physiology, cognition, and risk for schizophrenia by altering glutamate neurotransmission. However, the genetic data implicating *GRM3* as a susceptibility gene for schizophrenia remain weak, although the findings of Egan and colleagues [116] clearly suggest that further study is warranted.

9.6 FUNCTIONAL IMPLICATIONS OF SUSCEPTIBILITY GENES

In our view the evidence now strongly implicates *DTNBP1* and *NRG1* as susceptibility genes for schizophrenia, while the data for *DAO*, *DAOA*, *DISC1*, and *RGS4* are promising but not yet compelling. Even in the most convincing cases, the risk haplotypes appear to be associated with small effect sizes [odds ratio (OR) < 2.5] and, although this is difficult to determine, do not appear to fully explain the linkage findings that prompted each study. This could suggest that the associated polymorphisms/haplotypes are only in weak LD with the true pathogenic variants, that the linkages reflect variation at more than one susceptibility site in the same gene (or in multiple genes in the area of linkage), or that in some cases, despite the statistical evidence, the associations are spurious. Work in this area highlights how difficult it can be to determine what comprises a clear replication of associations that are based on LD, especially those based upon associations with haplotypes rather than single markers [117]. It is by no means certain that support requires the same pattern of association to be obtained or, conversely, that a negative finding can be regarded as a failure to replicate only if the associated allele or haplotype from the original study is examined. Detailed follow-up studies, including de novo mutation detection and detailed genotyping in large samples drawn from different populations, with the aim of answering these questions are now required.

For most geneticists, the purpose of disease gene identification is to enhance our understanding of pathogenesis. Thus it is now important that we identify the specific mechanisms by which the recently implicated genes alter the risk of schizophrenia and the molecular and cognitive processes that link these primary events to psychopathology. Already, it has been noted that several of the genes encode proteins that potentially impact on the function of glutamatergic synapses which might therefore be the location of the primary abnormality [118–120]. The possible importance of synaptic abnormalities in schizophrenia had already been recognized [121] and the recent genetic data suggest that there might at last be convergence between the genetics of schizophrenia and its neurobiology. However, there are several reasons for remaining cautious. First, the genetic evidence is not yet definitive and we have not identified the specific pathogenic variants, much less the pathogenic

mechanisms. Second, if we consider the two best-supported genes, *NRG1* encodes proteins with multiple functions of potential relevance to alternative hypotheses of schizophrenia, for example, aberrant myelination [50] while the function(s) of dysbindin are still obscure. Third, the widely held assumption that schizophrenia is a heterogeneous disorder with more than one core pathophysiology may well be correct. If this is so, then attempts to fit the data into a unified theory, while attractive and parsimonious, will ultimately prove futile. It is therefore vital that there is no letup in the hunt for novel schizophrenia genes, the finding of which will allow us to test existing and to generate novel hypotheses of pathogenesis.

9.7 CONCLUSIONS

Molecular genetic studies of schizophrenia are built upon the firm foundations of reliable diagnostic methodology and a wealth of genetic epidemiological data. The fact that most, if not all, disease genes apparently have only moderate or small effect sizes has proved challenging, as it has in the study of other common diseases. However, the sample and technological resources combined with the reagents generated by the Human Genome Project have begun to permit what appears finally to be genuine progress. A number of potential regions of linkage and two associated chromosomal abnormalities have been identified, and accumulating evidence favors several positional candidate genes, although in no case has the causative variant(s) been identified. These findings suggest that the positional genetic approach to schizophrenia is at last bearing fruit. As in most respects the task of positional cloning is becoming simpler, there are grounds for considerable optimism that genetics will continue to provide crucial insights into the aetiology of schizophrenia.

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POSTMORTEM BRAIN STUDIES: FOCUS ON SUSCEPTIBILITY GENES IN SCHIZOPHRENIA

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10.1	Introduction	343
10.2	Approaches to Identify Susceptibility genes in Schizophrenia	344
10.2.1	<i>COMT</i>	347
10.2.2	<i>DTNBP1</i>	348
10.2.3	<i>GRM3</i>	349
10.2.4	<i>DISC1</i>	349
10.2.5	<i>NRG1</i>	350
10.2.6	<i>GADI</i>	350
10.2.7	<i>RGS4</i>	351
10.3	Molecular Interactions of Schizophrenia Susceptibility Genes	351
10.3.1	<i>DISC1</i>	351
10.3.2	<i>DTNBP1</i>	352
10.3.3	<i>NRG1</i>	353
10.4	Conclusions	355
	References	356

10.1 INTRODUCTION

Schizophrenia is a syndrome characterized by psychotic symptoms (hallucinations, delusions, thought disorder) and cognitive impairment, with a prevalence rate approaching 1% worldwide. Over 100 years of neuropathology research has failed to identify a pathomnemonic lesion in the brains of schizophrenic patients. Nevertheless, important facts dictating the direction of a number of neuropathological studies have arisen from research efforts in related areas.

First and foremost is the evidence that schizophrenia has a clear genetic component. Results from twin and adoption studies show a heritability estimate

for schizophrenia of 70–90% [1–3]. However, analysis of recurrence risk estimates in families with one or more affected individuals clearly argues against schizophrenia being a single-gene disorder even with the possibility of incomplete penetrance [4]. Alternatively, the mode of transmission for schizophrenia is complex and multifactorial with the possibility of a number of genes conferring varying degrees of susceptibility. With this in mind, efforts have been directed at identifying allelic variants in genes that may confer increased risk for schizophrenia.

The identification of allelic variation in susceptibility genes that increase the risk for schizophrenia is only a first step in elucidating the pathophysiology. Exactly how susceptibility genes lead to schizophrenia remains largely unknown. A neurodevelopmental hypothesis argues that schizophrenia is the result of in utero events that lead to changes in neurogenesis, neuronal migration, and synaptic plasticity [5]. An alternative hypothesis of neurodegeneration proposes that schizophrenia is the result of neuronal loss or gliosis that occurs during aging [6]. For the most part, the latter theory has languished in part due to studies showing that such pathological hallmarks are only found in schizophrenia associated with dementia [6]. Still a third line of inquiry suggests that schizophrenia is a progressive developmental disorder with aberrant neuronal pruning during development that continues into early adulthood [7]. A better understanding of the pathophysiology associated with schizophrenia susceptibility genes may elucidate which, if any, of these hypotheses is correct.

Studies on the neurochemical basis of schizophrenia implicate many neurotransmitter systems, including dopamine, glutamate, γ -aminobutyric acid (GABA), and acetylcholine. During the last five decades, the dopamine system has been the central focus of schizophrenia research. The focus on dopamine relies largely on the fact that antipsychotics used to treat schizophrenia correlate with their ability to block D_2 receptors [8, 9]. A revised “dopamine hypothesis” posits that a hypofunction of the D_1 receptors in the prefrontal cortex (PFC) coexist with increased subcortical dopamine associated with D_2 receptors [10–12]. An alternative hypothesis based on the psychomimetic properties of phencyclidine (PCP), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, has implicated the glutamate neurons and is supported by neuroimaging and postmortem studies [13, 14]. A number of other postmortem studies implicate GABA inhibitory neurons in the neuropathology of schizophrenia [15–18]. Last but not least, both muscarinic (M) and nicotinic acetylcholine receptors [19] have also been postulated in the pathophysiology of schizophrenia [20, 21]. Again the identification of schizophrenia susceptibility genes may be the best evidence that multiple neurotransmitter systems are implicated in schizophrenia.

Identification of allelic variation in genes that increase risk for schizophrenia may implicate specific neurotransmitter systems and pathological processes. Moreover, this knowledge may elucidate molecular pathways involved in the pathophysiology of the syndrome that may alter both our diagnostic systems and lead to new therapeutic targets.

10.2 APPROACHES TO IDENTIFY SUSCEPTIBILITY GENES IN SCHIZOPHRENIA

With the high heritability estimates for schizophrenia, genetic researchers have postulated that there are a number of susceptibility genes, each conferring increased

risk for the disorder. The search for the genetic component of schizophrenia has primarily employed linkage analysis and association studies.

The aim of linkage studies is to identify regions of the genome that are cotransmitted with the disease in the affected individual but not in unaffected family members. Using polymorphic DNA markers distributed along each chromosome it is determined which DNA marker is cotransmitted with the disease due to its close physical proximity to the disease-causing gene. Once a candidate chromosomal locus harboring the disease gene is identified, fine mapping of this putative disease-linked region is then performed in order to pinpoint the disease-causing gene. Although this technique has been used with great success in identifying distinct chromosomal regions in the genome that may play a role in schizophrenia (Table 10.1), there are a number of limitations to linkage analysis. Due to the lack of a large number of

TABLE 10.1 Schizophrenia Susceptibility Genes Identified by Linkage Analysis and Association Studies

Gene	Location	Linkage Studies	Association Studies
<i>COMT</i>	22q11	[194]	[44] [41] [39] [195] [42]
<i>GRM3</i>	7q21–22	[99]	[106, 196] [197]
<i>DTNBP1</i>	6p22.3	[198] [199] [200] [201]	[202] [203] [204] [205] [93]
<i>DRD2</i>	11q23.2	[200] [206] [207]	[82] [77, 79, 80]
<i>DISC1</i>	4q42.2	[99, 208] [209] [149] [210] [211]	[212] [213] [214]
<i>NRG1</i>	8p21–22	[179, 215]	[216–219]
<i>AKT1</i>	14q32.33	[220] [221]	[96] [222]
<i>GAD1</i>	2q31	[202]	[223]
<i>RGS4</i>	1q23.1	[157]	[138] [224] [225–227] [228]

families with multiple affected individuals, ascertainment differences, broad phenotypic definitions, ethnic and environmental variations, and sparse polymorphic DNA markers, it is not surprising that linkage studies have not been an unmitigated success in the identification of schizophrenia susceptibility genes [22].

Chromosomal location by linkage analysis and fine mapping of the region followed by association studies of the genes present in this chromosomal region are designed to identify alleles of certain genes that are more common among individuals with the disorder than in the general population. Association studies are typically carried out by selecting specific polymorphisms in candidate genes that are thought to be correlated with the disease based on location and/or function and then determining the allelic frequency in affected versus unaffected individuals. Although useful, association studies are limited by the fact that the actual disease-causing polymorphisms have to be identified and then tested in affected versus unaffected individuals [23]. Since little is known about the neurobiology of complex mental disorders, picking truly relevant causative genes for association studies is inherently challenging. This difficulty is further increased when interpreting polymorphisms in noncoding regions that are not ultimately translated to a protein product. Moreover, inadequate patient–control matching, small sample size, and differences in allelic frequencies between different populations further contribute to the observed discrepancies in findings on the same gene by different groups.

Another method used to identify disease genes is analysis of cytogenetic abnormalities found more often in patients than in unaffected individuals. These chromosomal abnormalities such as translocations and large-scale deletions/duplications may be helpful if the affected chromosomal region harbors genes that contribute to the disease when their function is disrupted. Although there have been numerous reports on chromosomal anomalies associated with schizophrenia [24, 25], only two have provided convincing evidence that is supported by linkage data. The first is the identification of *DISC1* by a balanced reciprocal translocation [(1;11)(q42;q14.3)] in a large Scottish family with multiple individuals affected with schizophrenia and other psychiatric disorders [26, 27]. The second finding of interest is the association of schizophrenia with velocardiofacial syndrome (VCFS), which is caused by interstitial deletions of chromosome 22q11. In addition to a large number of physical anomalies associated with VCFS, patients with this disorder have a dramatic increase in the risk of psychosis, particularly schizophrenia [27–31]. It has been hypothesized that the deleted chromosomal region in VCFS may harbor schizophrenia susceptibility genes.

As is evident, over the last decade a large number of association studies have implicated numerous genes in the pathophysiology of schizophrenia. As an alternative to discussing all of these implicated genes in-depth, for the purpose of this postmortem brain studies review, we will concentrate on genes that fulfill the following criteria: First, the gene should be located on a chromosomal region implicated by linkage analysis. Second, there should be at least three positive association studies with the gene and schizophrenia in ethnically homogeneous populations [32]. Third, there should be data from postmortem molecular studies on expression of messenger RNA (mRNA) or protein available for the gene, particularly from regions of the brain implicated in schizophrenia. Finally, the gene product should have some functional significance for schizophrenia either at the time of neuronal development or in adult brain; that is, it would be expected to relate to prevalent schizophrenia hypotheses such as the neurodevelopmental hypothesis and

dopamine hypothesis. Based on these criteria we will review the following genes in detail: *COMT*, *DTNBP1*, *GRM3*, *DISC1*, *NRG1*, *GAD1*, and *RGS4*. Some of the linkage and association studies implicating these genes are listed in Table 10.1.

10.2.1 *COMT*

Catechol-O-methyl transferase (*COMT*) is a 27-kb gene located on chromosome 22q11 and encodes for a protein crucial for the enzymatic degradation of dopamine. This gene is composed of six exons: exons 1 and 2 are noncoding while exon 3 contains codons for two distinct promoters giving rise to two functionally different transcription products [33]. The short, 1.3-kb human mRNA produces the soluble isoform (S-COMT) while the longer 1.5-kb human mRNA produces the membrane-bound isoform (MB-COMT) [34]. Of these, the MB-COMT predominates in the human brain while S-COMT is found peripherally [33, 35, 36]. In the human brain, COMT mRNA is highly expressed in the prefrontal cortex, striatum, and midbrain [35] while electron microscopy studies have revealed the protein to be present on dendrites in these regions [37, 38].

A number of single-nucleotide polymorphisms (SNPs) in the *COMT* gene have been found to be associated with schizophrenia [39–43]. Only two of these polymorphisms affect COMT enzymatic activity [36]. A P₂ promoter SNP may have an effect on COMT expression as reflected in enzymatic activity in lymphocytes [36].

By far, the SNP with the most effect on the COMT expression and enzymatic activity is a missense mutation in exon 4 of the *COMT* gene yielding a nonsynonymous amino acid change from Val-Met at either position 108 in S-COMT or 158 in MB-COMT [44]. Although the *val* allele is associated with lower mRNA [40] and protein expression [36], it has higher enzymatic activity than the *met* allele [36]. Earlier studies of COMT enzymatic activity in brain found no differences between schizophrenics and controls [45, 46]. More recent postmortem findings also indicate that mRNA and protein expression levels of COMT are unaltered in the dorsolateral prefrontal cortex of schizophrenics [35, 36, 47]. However, an altered mRNA distribution pattern is observed in this region in schizophrenics when compared to controls [35]. Since the difference in frequency of the Val/Met allelic variation between schizophrenic cases and controls is small (approximately 6%), a large number of postmortem brains ($n = 272$) would be necessary to observe a significant difference in the schizophrenics [36]. As a consequence it is possible that the postmortem studies mentioned above were underpowered to observe a significant difference in COMT between schizophrenics and controls.

As dopamine transporters are expressed in low levels in the PFC [48] and thus are thought to play only a small role in synaptic dopamine reuptake in the PFC [49, 50], COMT may be crucial in terminating dopamine neurotransmitter function in PFC. Moreover, those individuals with the homozygous *val* allele in COMT may have lower cortical dopamine levels. Low levels of dopamine in the PFC alters downstream signaling pathways and initiates the synthesis of dopamine subcortically by an observed increase in nigral tyrosine hydroxylase (TH) mRNA in postmortem tissue [51–59].

In addition to COMT and TH, the level of dopamine receptors (DRD2) have also been examined in postmortem schizophrenia in an effort to better understand the role dopamine plays in disease pathology. The psychomimetic effect of dopamine agonists

and antipsychotic antagonism at DRD2 [9, 60–62] led to numerous studies of the D₂ receptor in postmortem human brain of schizophrenics and controls. While many studies have reported alterations in the level of D₂ receptors in postmortem tissue [63–68], much of the earlier findings have been challenged by the lack of isoform-specific antibodies and receptor-specific ligands as well as being confounded by antemortem neuroleptic treatment. Even in vivo neuroimaging receptor studies in drug-naïve patients, which report an increase of D₂ receptors in the striatum [69], have not been confirmed by others [70–73]. Currently, there is a general consensus from postmortem and neuroimaging studies that D₂-like receptors, particularly the D₂, are increased in the striatum [74, 75], although the etiology of this increase is probably secondary to neuroleptic treatment. A number of association studies have linked DRD2 with schizophrenia [76–82]; however, due to methodological problems in population stratification, the validity of these studies remains doubtful.

10.2.2 *DTNBP1*

DTNBP1 is a 140-kb gene located on 6p22.3, one of the best-established regions to have emerged from linkage studies of schizophrenia (Table 10.1). *DTNBP1*, is composed of 10 exons and encodes dysbindin 1 protein. Dysbindin mRNA is expressed widely in the brain and has been detected in the frontal and temporal neocortices, hippocampus, caudate, putamen, nucleus accumbens, amygdala, thalamus, and midbrain of the adult human brain [83]. These anatomical expression data are salient because regions such as frontal cortex and hippocampus are strongly implicated in schizophrenia.

Although the precise molecular function of dysbindin protein in the brain has not been determined, dysbindin binds to dystobrevins, which are components of the dystrophin-associated glycoprotein complex (DCG) [84]. DCG is a multiprotein complex that is required for normal functioning of muscle cells, and several DCG-like complexes have been identified in postsynaptic densities in the brain [85]. Alterations of these complexes have been implicated in the cognitive impairment that commonly occurs in patients with Duchenne muscular dystrophy (DMD) [86, 87]. Interestingly, the absence of dystrophin in the *mdx* mouse model of DMD results in altered distribution of dysbindin in the cerebellum [88]. Dysbindin has also been found to be a component of the biogenesis of lysosome-related organelles complex (BLOC)-1 [89], which may play a role in schizophrenia and will be subsequently discussed in greater detail.

Postmortem brain studies have found expression levels of dysbindin mRNA levels to be reduced in the dorsolateral prefrontal cortex of schizophrenics compared to controls [83], and SNPs in three prime untranslated region (3'UTR), intron 3, 5'UTR, and 5' flanking region significantly impact dysbindin mRNA levels in PFC [83]. Dysbindin protein levels are also significantly reduced in the postmortem hippocampus of schizophrenic patients [90]. Dysbindin mRNA levels in human brains are reduced by cis-acting polymorphisms, and a schizophrenia risk haplotype was also found to be associated with reduced *DTNBP1* expression in the frontal, parietal, or temporal cortices [91, 92].

Downregulation of dysbindin expression has been found to cause reduced phosphorylation of AKT1 [93]. The serine–threonine protein kinase gene encoded by AKT1 is 32.8 kb in size, located on chromosome 14q32 spanning 14 exons.

AKT1-GSK-3 β (glycogen synthase kinase 3 β) is a target of lithium and has been implicated in bipolar disorder [94]. In the developing nervous system AKT1 is a critical mediator of growth factor-induced neuronal survival. In situ histochemistry of mouse brain during normal development determined that embryonic AKT1 mRNA levels were high throughout the entire neuroaxis. The level of expression gradually decreased during postnatal development and into adulthood [95]. AKT1 protein levels are reduced in lymphocytes, hippocampus, and PFC of schizophrenics [96]. Reduced phosphorylation of GSK-3 β , which is a target of AKT1, was also detected in lymphocytes and frontal cortex lysates [96]. GSK-3 β mRNA levels are also reduced in dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients versus controls [97]. Based on these findings, AKT1 may play a role in neurodevelopment and altered postnatal levels of AKT1 and its downstream molecular targets may contribute to increased risk for schizophrenia.

10.2.3 *GRM3*

GRM3 (human metabotropic glutamate receptor subtype 3) is mapped to chromosome 7q21–22 [98] and is considered a positional and functional candidate gene for schizophrenia based on a genomewide scan [99]. *GRM3* is approximately 220 kb in size and contains six exons [100]. The protein product of the gene is assembled into a membrane-bound receptor coupled to second-messenger pathways and allows for a decrease in glutamate release [101, 102]. *GRM3* is widely distributed in the DLPFC, thalamus, and hippocampus. However, neither the mRNA nor protein expression was altered in schizophrenics relative to controls in any of these regions [103–105]. In our laboratory, in vivo functional magnetic resonance imaging (MRI) testing has revealed that an intronic SNP in the *GRM3* gene is associated with poor hippocampal and prefrontal functions [106]. This study also reports that the same intronic SNP is associated with a lower mRNA expression in the postmortem brain tissue of the excitatory amino acid transporter 2 (EAAT2), a modulator of synaptic glutamate levels. Additionally, a number of splice variants of *GRM3* have been detected in the human brain [107].

10.2.4 *DISC1*

DISC1 is a 414.3-kb gene located on chromosome 1q42.2 and consists of 13 exons. As mentioned above, *DISC1* was originally identified as a candidate gene for schizophrenia in a large Scottish family, in which a balanced translocation involving chromosomes 1 and 11 was strongly linked to schizophrenia, schizoaffective disorder, bipolar affective disorder, and recurrent major depression [26]. In this family, carriers of the translocation were found to have reduced P300 amplitude, which is observed in some patients with schizophrenia [27]. Subsequent association studies identified a number of polymorphisms in the *DISC1* gene associated with schizophrenia and affective disorder (Table 10.1).

In the adult mouse brain, *DISC1* is expressed widely including the olfactory bulb, cortex, hippocampus, hypothalamus, cerebellum, and brain stem. During development, *DISC1* protein is detected at all stages, from embryonic day 10 (E10) to 6 months old, with two significant peaks of protein expression of one of the *DISC1* isoforms at E13.5 and postnatal day 35 [108]. Interestingly, these time points

correspond to periods of active neurogenesis and puberty in the mouse. These results suggest that *DISC1* may play a critical role in brain development, lending support to the neurodevelopmental hypothesis of schizophrenia [108]. Although the precise function of *DISC1* in the brain is unknown, a number of *DISC1*-interacting partners have been identified, including FEZ1, NUDEL, and LIS1, which are known to play a role in neuronal development and functioning. Postmortem studies have detected altered subcellular distribution of *DISC1* in patients with psychosis and alcohol/substance abuse, with increased ratios of nuclear to cytoplasmic *DISC1* protein levels in patients [109]. Although *DISC1* mRNA expression is unchanged in postmortem human brains of patients with schizophrenia, the expression of *DISC1*-interacting proteins NUDEL, FEZ1, and LIS1 mRNA is significantly reduced in schizophrenic tissue in both the DLPFC and hippocampus [110]. Altered interactions between *DISC1* and its binding partners have also been investigated in order to understand more accurately the biology of *DISC1* as a schizophrenia susceptibility gene.

10.2.5 *NRG1*

Neuregulins are a family of widely expressed growth and differentiation factors. The neuregulin gene family consists of four genes (*NRG1* to *NRG4*). *NRG1* is approximately 1.125 Mb in size and contains at least 21 exons and 9 potential promoters [111]. Thus far six distinct isoforms and at least 16 splice variants have been reported [111, 112]. While *NRG2*, *NRG3*, and *NRG4* are found only in adult tissue, *NRG1* type I isoform is expressed early in development, type II is expressed late in development, and type III is expressed in peripheral sensory and motor neurons as well as in the brain [113]. The newly discovered isoform types IV–VI [111] have an unknown anatomical distribution and functions. In the developing and adult human brain, *NRG1* mRNA and protein are present in the hippocampus, cerebellum, neocortex, and some subcortical nuclei [114]. *NRG1* type I expression is increased in the DLPFC of schizophrenic patients, while the type II and III levels remain unchanged [115]. Recently, our laboratory has found 5' SNPs in the *NRG1* gene that are associated with a change in the mRNA expression of type I and IV isoforms [116]. Postmortem microarray studies also report a significant reduction in the level of ErbB3 receptors, through which neuregulins indirectly exert their biological effect, in the prefrontal cortex of schizophrenics [117, 118]. However, preliminary in situ hybridization studies done in our laboratories using an ErbB3 probe could not confirm the previous findings [119].

10.2.6 *GAD1*

Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme that converts glutamic acid to GABA, an inhibitory neurotransmitter in the brain. *GAD1* gene is 45 kb in size, contains 16 exons [120], and is mapped to chromosome 2q31 [121]. Alternative splicing of this gene results in two products, the predominant 67-kD form and a less abundant 25-kD form. GAD67 is concentrated in interneurons and neurons that fire tonically [122] and is involved in the nonvesicular release of GABA [123].

A number of postmortem studies have reported reduced GAD67 mRNA and protein in PFC of schizophrenic patients [15, 17, 18]. On the contrary, GAD67

mRNA was found to be unaltered in the hippocampus of schizophrenic patients [124]. However, one study has found an increase in GAD67 mRNA in the DLPFC [125]. Besides alterations in GAD67, ligand binding and autoradiography studies demonstrate that GABA_A receptors are upregulated in the cingulate cortex, PFCs and hippocampus of schizophrenics [126], while the short isoform of GABA_A γ subunit is downregulated in the DLPFC [127]. This, in addition to other studies on GABA_A receptor binding and immunoreactivity [16, 18, 128–135], provides strong evidence for an overall hypofunction of the GABAergic system.

10.2.7 *RGS4*

Regulator of G-protein signaling 4 (*RGS4*) is a 110-kb gene located on chromosome 1q23.1 spanning 16 exons. *RGS4* is involved in neuronal differentiation and is under dopaminergic regulation [136, 137]. Using microarray analysis *RGS4* mRNA expression was found to be reduced in the PFC in schizophrenics and controls [138]. These studies were confirmed by in situ hybridization in PFC [138]. Post-mortem studies on expression of brain *RGS4* mRNA levels detected highest levels in the cortical layers, with moderate levels observed in the parahippocampal gyrus. Inner layers of the frontal cortex, which is implicated in schizophrenia, also showed dense labeling [139]. Given these findings, *RGS4* continues to be an interesting candidate gene for schizophrenia.

10.3 MOLECULAR INTERACTIONS OF SCHIZOPHRENIA SUSCEPTIBILITY GENES

As discussed in the previous sections, the neuropathology of schizophrenia involves a number of susceptibility genes. Many of these susceptibility genes, alone, may exert only a small effect on the disease pathology but working via molecular partners may converge to share a common pathway leading to disease. In the past, much of the attention had been focused on convergent anatomical pathway involving neurotransmitters such as dopamine, glutamate, and GABA. In this section, our attention is focused on those susceptibility genes that have been shown to interact through molecular partners in animal models or artificial in vitro systems. This will then be followed by our hypotheses of how these interactions may play a role in schizophrenia with evidence from postmortem studies whenever available.

10.3.1 *DISC1*

As mentioned earlier, *DISC1* was identified by a chromosomal translocation in a large Scottish family and is considered a susceptibility gene for schizophrenia by a number of association studies. In an effort to understand the cellular function of *DISC1*, yeast-two hybrid studies have been used to identify molecular interactors of *DISC1*. It was found that *DISC1* has numerous binding partners, including NUDEL, FEZ1, ATF4/5, MAP1A [140–142]. NUDEL is a component of a pathway involved in cytoplasmic dynein movement and is involved in neurofilament assembly, neuronal migration, and development of neurite morphology [143–148]. Overexpression of truncated *DISC1* protein inhibits neurite outgrowth in PC12 cells, suggesting

that the DISC1–NUDEL complex may be involved in neuronal outgrowth [141, 148, 149]. The predicted peptide product resulting from the Scottish translocation removes the interaction domain for NUDEL. The defective DISC1–NUDEL complex may be a cause of neurodevelopmental abnormalities in schizophrenia [150]. Recently, it has been shown that NUDEL oligopeptidase activity is under tight regulation through binding to DISC1 since a mutation very close to the DISC1 binding site of NUDEL abolishes this activity [151]. Interestingly, NUDEL cleaves a number of neuropeptides in vitro, some of which have previously been implicated in the pathophysiology of schizophrenia, including neurotensin (NT) (reviewed in [152, 153]). Postmortem studies have revealed increased NT levels in the frontal cortex [154] and decreased NT binding in the entorhinal cortex in schizophrenic patients compared to controls [155]. NT receptor agonists may be potential antipsychotics; thus inhibition of NUDEL could lead to increase in local concentration of NT, which may have an antipsychotic effect [151]. Cell culture studies in cortical neurons have found evidence that DISC1 may colocalize with mitochondrial markers and that its subcellular targeting is independent of the NUDEL binding site [149]. Hayashi et al. have also demonstrated that DISC1 and NUDEL bind in a neurodevelopmentally regulated manner and form a trimolecular complex with another protein, lissencephaly 1 (LIS1). LIS1 is involved in neuronal migration and corticogenesis. Although the function of this complex is currently unknown, it is thought to play a role in dynein-mediated motor transport [151].

Another interacting partner of DISC1 is the fasciculation and elongation protein zeta-1 (FEZ1), which is a mammalian homolog of the *Caenorhabditis elegans* UNC-76 protein, involved in axonal outgrowth and fasciculation. Miyoshi et al. demonstrated that DISC1 participates in neurite extension through its C-terminal interaction with FEZ1 [156]. The chromosomal location for FEZ1 was previously implicated in a schizophrenia linkage analysis, although results from different populations vary in significance [157]. A modest association between schizophrenia and FEZ1 polymorphisms has been detected in a subset of Japanese patients [158].

An interesting hypothesis is that altered expression of DISC1 and/or its molecular partners NUDEL, FEZ1, and LIS1 may underlie its pathogenic role in schizophrenia and explain its genetic association [110]. Although DISC1 mRNA expression is unchanged in postmortem human brains of patients with schizophrenia and there is no association with previously identified risk SNPs, the expression of NUDEL, FEZ1, and LIS1 mRNA is significantly reduced in schizophrenic tissue in both the DLPFC and hippocampus and the expression of each gene showed association with a high-risk DISC1 polymorphism [110]. These data implicate genetically linked abnormalities in the DISC1 molecular pathway in the pathophysiology of schizophrenia. Given its role in brain development and plasticity via its interaction with a number of different proteins, *DISC1* remains a candidate gene for schizophrenia and an understanding of its exact mechanistic role in neuronal pathways may shed more light on the disease.

10.3.2 *DTNBP1*

Over the last decade the involvement of *DTNBP1* gene, which encodes dysbindin protein, with schizophrenia has been demonstrated by a large number of association studies and several postmortem findings. However, only recently have we begun to

understand the role of dysbindin in the brain and the biological pathways that may be affected by disruption of *DTNBP1* function.

Dysbindin overexpression and knockdown experiments using rat cortical cells suggest that dysbindin regulates the expression of synaptosomal-associated protein 25 (SNAP25) and synapsin I proteins in the presynaptic machinery [93, 158]. SNAP25 is a molecular component of the SNARE (soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptors) protein complex, which is involved in intracellular vesicle trafficking and neurotransmitter release [159]. Synapsin I is localized to synaptic vesicles that are both docked and located away from the plasma membrane [159]. Reduction in protein levels of SNAP25 in the frontal cortex [160] and synapsin I in the hippocampus [161] has been reported in patients with schizophrenia, although other studies have found no change in SNAP25 [162]. Dysbindin also influences extracellular glutamate levels and glutamate release [93]. As discussed earlier, hypofunction of the glutamatergic neurotransmitter system has been implicated in schizophrenia [163].

Numakawa et al. reported that overexpression of dysbindin protected cortical neurons from cell death upon serum deprivation [93, 163]. This effect was mediated through the phosphoinositol 3 (PI3)–kinase–AKT signaling pathway. Downregulation of dysbindin expression caused reduced phosphorylation of AKT [93]. Independently, impaired PI3–kinase–AKT signaling in schizophrenia has also been observed in the PFC [96]. Reduced dysbindin expression in the schizophrenic brain [83, 90] may contribute to the impairment in AKT signaling. Disrupted dysbindin–AKT signaling might cause increased cell vulnerability and neuronal loss in vulnerable brain regions leading to the onset of schizophrenia symptoms [93]. These data establish a role for dysbindin in regulating neuronal cell viability.

Biogenesis of lysosome-related organelles complex-1 (BLOC1) is a ubiquitously expressed aggregation of interacting proteins required for the biogenesis of specialized lysosome-related organelles. This complex is linked to the secretory and endocytic pathways for cellular protein and lipid trafficking [164]. BLOC1 is comprised of several interacting proteins, including pallidin, muted, cappuccino, and dysbindin. Several of the genes that make up the BLOC1 complex are defective in the genetic disorder called Hermansky–Pudlak syndrome (HPS) [165–167]. Mutations in dysbindin cause a form of HPS called HPS7 [89]. A mouse model for HPS, the Sandy mouse, has a deleted *DTNBP1* gene and expresses no dysbindin [89]. This mouse model could be a powerful tool for investigating the function of dysbindin in the brain in vivo. Although the precise biological role of dysbindin in the BLOC1 complex is still unknown, it might be involved with endocytic vesicle docking and fusion. Presynaptic protein expression, glutamate release, AKT phosphorylation, and neuronal viability may also be assessed in vivo using this mouse model.

10.3.3 *NRG1*

Rodent studies have shown *NRG1* to function in a converging neurochemical pathway with glutamatergic, cholinergic, and GABAergic receptor systems [168–172]. As molecules located intracellularly, membrane bound, and released into the synapse, neuregulins exert their effects by acting as ligands for three receptors, ErbB2, ErbB3, and ErbB4, which are tyrosine kinases belonging to the epidermal

growth factor (EGF) receptor-related family [173]. The ErbB4, in particular, has been shown to be associated with postsynaptic density-95 (PSD-95) and NMDA receptors [174]. PSD-95, like the NMDA receptor subunits, is altered in schizophrenia [175–177] and is reported to enhance NRG1 signaling by ErbB4 dimerization [178]. Additional evidence for a molecular interaction between NRG1 and NMDA receptors has been shown in cerebellar granule cell culture, where the more biologically active β_1 isoform of NRG1 increases the levels of the NR2C subunit of the NMDA receptors [169]. More recently, the same isoform has been shown to cause ErbB dimerization resulting in increased intracellular Ca^{2+} and activation of extracellular regulated kinase (ERK) in the pyramidal neurons of the rat PFC. This downstream cascade is then thought to enhance the actin depolymerization, leading to internalization of NR1 subunits, downregulation of NMDA receptors, and a decrease in NMDA receptor-mediated current [168]. Mice lacking one copy of the *NRG1* gene showed decreased NMDA receptor binding and behavioral features related to schizophrenia [179]. These results are suggestive of the hypothesis that polymorphisms in the *NRG1* gene may lead to deficits in glutamate signaling, offering an explanation of the concept of glutamate hypofunction in schizophrenia.

Neuregulins were initially discovered as acetylcholine receptor inducing activity at the neuromuscular junction, but less is known about their effect on brain nicotinic acetylcholine receptors (nAChRs). Both high- and low-affinity nAChRs are reported to be decreased in the hippocampus, thalamus, and PFC of schizophrenics [19, 180–184]. In cultured hippocampal neurons, the NRG1 isoform β_1 was shown to increase presynaptic neurotransmitter release and to increase the number of surface membrane α_7 nAChRs [171]. Although the exact mechanism is unknown, the upregulation of nAChRs by NRG1 is presumed to occur via intracellular cascade involving ErbB4 receptors due to the colocalization of both of these receptors onto GABA interneurons in the hippocampus [174, 178, 185]. Another isoform of NRG1, the type III, is a membrane-bound bidirectional molecule which is thought to also alter nAChRs. When the extracellular portion of this isoform binds ErbB4 receptors, an intracellular cascade occurs in both the NRG1- and ErbB4-expressing neurons. Back signaling, which occurs in the NRG1-expressing neuron, is accompanied by proteolytic release and translocation of NRG1 intracellular domain into the nucleus, leading to changes in gene expression and resistance to apoptosis [186]. This back signaling in sensory neurons stimulates a redistribution of the nAChR α_7 from a diffuse somatodendritic location to a punctate axonal distribution [187]. In support of this, heterozygous mice of type III isoform have deficits in prepulse inhibition with a decrease in α_7 -subunit binding [188]. Concurrently, our own postmortem studies lend support for the interaction of NRG1 and nAChRs by the finding that 5' NRG1 SNPs identified in the original disease haplotype [179] are associated with altered α_7 -subunit receptor binding in the human DLPFC [189].

The immunoglobulin (Ig) NRG isoform has been shown to increase levels of the β_2 -subunit protein of the GABA receptors in cerebellar granule cell culture [172]. Furthermore, binding of NRG1 with ErbB4 receptor kinase is necessary for upregulation of GABA receptors [172, 190]. Because the β_2 subunits are responsible for targeting the whole receptor to the cell surface [191, 192], Rieff and colleagues [172] suggest that NRG1 may be responsible for the total number of functional receptors at the postsynaptic terminal. In contrast, infusion of the Ig isoform of NRG1 decreases the mRNA expression of the GABA_A α subunits in hippocampal

slices [193]. This report suggests that NRG1 may cause a downregulation of the GABAergic synaptic activity. The variability in the regulation of receptor subunits reviewed above indicates the diversity of the functions served by NRG1 in various brain regions. Most importantly in schizophrenia, the search should be focused on methods to elucidate the function of NRG1 in those brain regions known to be involved, such as the hippocampus, DLPFC, and midbrain.

10.4 CONCLUSIONS

Schizophrenia is a devastating neuropsychiatric disorder the genetics of which has been under extensive investigation for several decades. Despite being an exceedingly complex disease in terms of both etiology and pathogenesis, recent research is finally shedding light on schizophrenia susceptibility genes. Linkage analysis using families with multiple affected individuals has identified regions of the genome that are cotransmitted with the disease and may harbor genes involved in the pathogenesis of schizophrenia. To narrow the chromosomal regions implicated by linkage analysis, association studies using candidate gene analysis have identified a number of genes that may increase susceptibility to schizophrenia. Postmortem brain studies of these genes (such as *COMT*, *DTNBP1*, *GRM3*, *DISC1*, *NRG1*, *GAD1*, and *RGS4*) suggest that there are at least four neuronal systems implicated in the pathophysiology of schizophrenia. (1) Genes such as *COMT*, *GRM3*, and *GAD1* involve dopamine, glutamate, and GABA neurons, and it is conceivable that convergent interactions between these genes may be abnormal in schizophrenia. (2) *DTNBP1*, which encodes dysbindin protein, is involved in the presynaptic machinery and influences extracellular glutamate levels. Dysbindin influences cellular secretory and endocytic pathways responsible for protein and lipid trafficking through its involvement with the BLOC1 protein complex. (3) *DISC1* and its binding partners *FEZ1*, *NUDEL*, and *LIS1* are involved in cytoplasmic dynein movement, neurofilament assembly, neuronal migration, and neurite morphology and may play a role in the neurodevelopmental deficits observed in schizophrenia. (4) Neuregulins, encoded by *NRG1*, are involved in the maintenance of synaptic plasticity by regulating excitatory (glutamate, ACH) and inhibitory (GABA) neurotransmitters, and a disruption in their function, particularly during development, may contribute to schizophrenia.

Although the precise neurobiological cause of schizophrenia continues to be unknown, the abundance of evidence regarding susceptibility genes for schizophrenia cannot be dismissed. Susceptibility genes may provide important insights into the pathogenesis of schizophrenia. Identification of the molecular and cellular mechanisms that link susceptibility genes to the neurobiological functioning of the brain continues to be a major focus of research. It is conceivable that of the multitude of genes expressed in the brain, some of the susceptibility genes identified would have an effect on one another by either direct molecular binding or indirect upstream functioning. As evidence for the functioning of the various susceptibility genes increases, it may be determined that these genes operate in a convergent molecular pathway affecting neural development and synaptic plasticity. The disruption of multiple genes within this pathway may lead to the development of schizophrenia. Such a convergent biochemical pathway may also be an attractive target for therapeutic intervention.

Despite tremendous advances in schizophrenia research, numerous questions remain unanswered. Future studies are needed to analyze the abundance, distribution, developmental expression patterns, and perhaps most importantly molecular function and abnormalities in schizophrenia susceptibility genes. Data from these studies will not only offer insights into the functions of numerous neurobiological pathways in healthy individuals but also provide a mechanistic link between the genetics and the neurobiology of schizophrenia and other mental illnesses. Further identification of new schizophrenia susceptibility genes, dissection of the molecular pathways involved, discovery of the underlying causative defect in the schizophrenic brain, and ultimately development of better therapeutic strategies are important future goals of schizophrenia research that can be advanced by the study of postmortem brains.

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11

PHARMACOTHERAPY OF SCHIZOPHRENIA

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11.1	Introduction	370
11.2	Neurochemical Hypotheses of Schizophrenia	371
11.2.1	Dopamine Hypothesis	371
11.2.2	Glutamate (NMDA Receptor Hypofunction) Hypothesis	373
11.2.3	Integration of Dopamine and Glutamate Hypotheses	374
11.3	Hypothesized Mechanisms of Action of Antipsychotics	376
11.3.1	D ₂ Receptor Occupancy and Antipsychotic Effect	376
11.3.2	High 5-HT _{2A} versus D ₂ affinity	378
11.3.3	D ₂ Occupancy Thresholds and Rapid Dissociation	378
11.3.4	Highly Selective D ₂ /D ₃ Antagonism, D ₄ Antagonism, and Regional Specificity	380
11.3.5	Role of D ₁ Receptors and Other Mechanisms	380
11.3.6	Synthesis	381
11.4	Clinical Profiles of Antipsychotic Drugs	383
11.4.1	First-Generation or Conventional Antipsychotics	383
11.4.2	Second-Generation or Atypical Antipsychotics	383
11.4.3	Safety and Tolerability	387
11.5	Drugs in Development and Future Directions	388
11.5.1	Drugs Acting Directly or Indirectly on Dopamine System	389
11.5.2	D ₄ Antagonists	390
11.5.3	D ₃ Antagonist	390
11.5.4	D ₁ Agonists and Antagonists	390
11.5.5	Neurotensin Agonist/Antagonist	390
11.5.6	Neurokinin Antagonists	391
11.5.7	Drugs Acting on Glutamate System	391
11.5.8	Noradrenergic Agents	393
11.5.9	Cholinergic Agents	393
11.5.10	Other Agents	394
11.6	Conclusion	394
	References	395

11.1 INTRODUCTION

It has been over half a century since effective treatment for psychosis was introduced into clinical practice. Chlorpromazine was the first of several antipsychotics, now called conventional or first-generation antipsychotics (FGAs), which revolutionized the treatment of psychotic disorders and enabled large numbers of individuals afflicted with these illnesses to be discharged from chronic care institutions and be cared for in the community. However, the high rate of extrapyramidal symptoms (EPSs) at therapeutic doses of these drugs was a major impediment to patient acceptability [1], and efficacy limitations such as minimal or no improvement of cognitive deficits, negative symptoms, and mood symptoms resulted in a substantial number of patients not tolerating these medications, not responding to them, or refusing to take them.

The second major step in the treatment of psychosis was the discovery of clozapine in 1958. Because of the risk of agranulocytosis, clozapine remained relatively obscure until the seminal study by Kane et al. [2] convincingly demonstrated its superior efficacy to chlorpromazine in treatment-resistant patients. Clozapine was reintroduced in the United States in 1990 under strict blood-monitoring requirements to mitigate the risk of agranulocytosis. Numerous studies have since confirmed its superior efficacy in patients with treatment-resistant schizophrenia [3, 4] and its broader spectrum of efficacy, including improvement in cognition [5], aggressivity [6], mood stabilization [7–9], and suicide risk reduction [10]. But perhaps the most important aspect of the clinical profile of clozapine is that it almost never causes parkinsonian-type side effects. For decades it was felt that the antipsychotic effect of antipsychotics and their propensity to induce parkinsonian-type side effects were inextricably linked; that is, to get an antipsychotic effect, it was necessary to increase the dose of the drug to the point of impairment of fine motor control. This, so-called neuroleptic threshold concept was essentially invalidated by the observed clinical profile of clozapine.

The “atypical” clinical profile of clozapine spurred the development of safer alternatives that would preserve the “atypicality” of clozapine and eliminate the agranulocytosis risk. The second-generation antipsychotics (SGAs), all introduced within the last decade or so, are also commonly referred to as atypical antipsychotics (risperidone, olanzapine, quetiapine, ziprasidone, and aripiprazole). Other agents not approved in the United States that have atypical properties include amisulpiride, zotepine, and sertindole (withdrawn from clinical use because of QT prolongation). Although there is debate as to what constitutes atypicality, the defining feature of this class of medications is the separation of the dose that results in a therapeutic effect from that which is associated with an increasing risk of EPSs. All SGAs meet this definition, but there are substantial differences in clinical profiles within the group. Unfortunately, the progress made in reducing motor side-effect burden with SGAs is tempered by their increased liability for causing weight gain (especially clozapine and olanzapine) and metabolic side effects, including increased risk of diabetes and dyslipidemia. The efficacy of these newer agents on psychotic symptoms has not been proven to be substantially different from conventional antipsychotics [11, 12], although one meta-analysis demonstrated a modest efficacy advantage for some of the SGAs [13]. The literature suggests incremental gains in cognitive function [14], relapse prevention [15–17], and negative symptoms [18, 19].

Despite the proven utility of antipsychotics in the management of schizophrenia, much remains to be deciphered about their differential clinical profiles, their mechanisms of action, and the pathophysiological substrates of the disease states in which they are used. In this chapter we will begin by briefly reviewing the two predominant theories of the proposed neurochemical dysregulation in schizophrenia; this will provide the conceptual framework for the rest of the chapter. We will then discuss and critique the various theories that attempt to explain the mechanism of action of antipsychotics, summarize the literature on their efficacy and side-effect profiles, and review strategies and targets for future drug development.

11.2 NEUROCHEMICAL HYPOTHESES OF SCHIZOPHRENIA

This review will cover the current status and the salient research relevant to the dopamine and glutamate dysfunction hypotheses of schizophrenia but is not meant to be an exhaustive review of the data. Readers are referred elsewhere for more detailed information [20–23; see also other chapters in this part].

11.2.1 Dopamine Hypothesis

The dopamine hypothesis of schizophrenia was proposed more than 40 years ago by Carlsson and Lindquist [24], who showed that haloperidol and chlorpromazine increased the turnover of brain monoamines as reflected by an increase in their metabolites. They proposed that these drugs acted on monoamine receptor targets in brain causing a secondary compensatory increase in monoamine turnover. Numerous studies have documented that dopamine-releasing drugs such as amphetamine are capable of inducing a paranoid psychotic state in normal control subjects that closely resembles paranoid schizophrenia [25, 26]. Additionally, psychotic symptoms are exacerbated in approximately 40% of schizophrenic patients after receiving smaller doses of such stimulants that are not psychotogenic in normals [27, 28]. Moreover, data suggest that patients who show such symptom exacerbation upon stimulant challenge are at increased risk for acute relapse if not taking antipsychotics [29–31]. In 1967 Van Rossum [32] was the first to hypothesize that the therapeutic effects of antipsychotics were related to their action on dopamine (DA) receptors. However, it was not until the mid 1970s that these receptors were actually identified and the actions of these drugs linked to D₂ receptors [33, 34]. Advances in functional brain-imaging [single-photon emission computerized tomography (SPECT) and positron emission tomography (PET)] technology in the past 20 years have provided in vivo evidence in support of the dopamine hypothesis, at least in the pathogenesis of psychotic symptoms.

Wong et al. [35] using [¹¹C]-*N*-methyl-spiperone were the first to study in vivo D₂ receptor density in the striatum of patients with schizophrenia and found a substantial elevation compared to normal control subjects. Most subsequent studies, however, failed to replicate this finding [23]. One factor frequently quoted as impacting the results in D₂ receptor quantification studies is the upregulation of these receptors following antipsychotic treatment, a fact well established in the animal literature. The usual drug-free period prior to imaging is two to three weeks, but it is not precisely known if this is enough time for complete reversal of

upregulation secondary to antipsychotic therapy. Other factors, such as small sample size, differences in receptor ligands used in different studies, and their susceptibility to competition from endogenous dopamine for the D_2 receptors, may also have contributed to these inconsistent results [23, 36, 37]. However, when all the PET and SPECT studies that have addressed this question are pooled, the effect size of increased D_2 receptor densities in schizophrenia was 0.51 ± 0.76 [standard deviation (SD)] [23]. The probability of yielding this effect size under the null hypothesis of “no difference” is <0.05 [23]. This translates to a 12% increase in D_2 receptor density parameters in patients with schizophrenia, an increase that is significant compared to controls, although not of the magnitude reported in postmortem studies [23].

The state of presynaptic DA synthesis in the striatum of patients with schizophrenia has also been investigated. Seven studies [38–44] have examined dopa-decarboxylase (a non-rate-limiting enzyme in the synthesis of DA) in patients with schizophrenia, and most demonstrated a higher accumulation of radioligand labeled 3,4-dihydroxy-L-phenylalanine (DOPA), suggestive of increased dopamine synthesis. Increased DA synthesis, however, does not necessarily imply increased DA release into the synapse. In a series of elegant experiments using SPECT and PET, Laruelle et al. [45] and Breier et al. [46] found that an intravenous amphetamine challenge in drug-naïve/drug-withdrawn patients with schizophrenia resulted in a greater *decrease* in radioligand binding relative to baseline as compared to normals, suggesting a relatively greater release of DA upon amphetamine stimulation in schizophrenia. As expected, the increase in DA release following amphetamine was associated with transient worsening of psychotic symptoms. Furthermore, the severity of baseline psychotic symptoms was correlated with baseline DA level as well as the magnitude of DA release upon amphetamine challenge and subsequent treatment response. However, the magnitude of DA release accounted for only 30% of the variability in the psychotic response [23]. Finally, higher challenge-induced DA release was found in most but not all patients in acute relapse and was not demonstrable in stable outpatients.

Although these seminal studies confirmed a dysregulation in the synaptic control of DA in at least some patients with schizophrenia in response to an exogenous pharmacological challenge, they did not provide information on the natural state of the DA synapse. Another productive strategy to indirectly assess intrasynaptic DA has utilized two PET studies to quantify D_2 receptor occupancy before and after two days of treatment with α -methylparatyrosine (AMPT), which acutely depletes DA; the extent of the *increase* in D_2 radioligand binding from baseline to post-AMPT treatment is an indirect quantitative measure of intrasynaptic DA concentration at baseline; that is, the greater the increase in D_2 binding following DA depletion, the greater the baseline occupancy of D_2 receptors by DA. Using this strategy in acutely relapsed medication-naïve or medication-free patients, Abi-Dargham et al. [47] found a statistically greater increase in D_2 binding following DA depletion in patients with schizophrenia compared to healthy age- and sex-matched controls. These observations suggest that in acute exacerbation schizophrenia is associated with higher intrasynaptic DA levels compared to normal controls. Psychotic symptoms improved upon DA depletion (cf. experience with reserpine in the 1950s), and the increase in radioligand binding in the striatum was correlated with the degree of improvement of psychotic symptoms. If these observations are confirmed, it would provide direct proof of the DA hypothesis that increased DA transmission is, at least in part,

responsible for psychotic symptoms [23]. It is important to reiterate that these conclusions of increased subcortical DA transmission in schizophrenia are based on group data and individual patients may fall within the normal range of DA transmission parameters.

Thus far we have focused on DA transmission indices in subcortical regions and their relation to psychosis. Another brain region implicated in the pathophysiology of schizophrenia, especially with regards to cognitive dysfunction and negative symptoms, is the dorsolateral prefrontal cortex (DLPFC) [48]. A few PET studies have examined the density of D₁ receptors in the prefrontal cortex (PFC), where this is the predominant DA receptor subtype. The results have been conflicting. Okubo et al. [49] demonstrated a *decrease* of D₁ receptors in the PFC, Abi-Dargham et al. [50] found an *increase* in D₁ receptors in the DLPFC, while Karlsson et al. [51] were unable to demonstrate a difference from normals in D₁ receptor density in any subcortical or neocortical region that was examined. Receptor ligand differences have been proposed as accounting for these discrepancies [52]. Interestingly, despite variable outcomes in D₁ receptor densities in the DLPFC in the above studies, all found an association between D₁ binding and various cognitive measures and/or negative symptoms. Potential D₁ receptor dysfunction is especially relevant for the pathophysiology of schizophrenia given the postulated role for this receptor in mediating critical aspects of cognitive function [53] and the ability of a full D₁-like agonist (ABT431) to reverse working memory deficits that emerge after long-term treatment with antipsychotics in nonhuman primates [54]. Functional brain-imaging studies in patients with schizophrenia have consistently demonstrated a hypometabolic state in the DLPFC which is correlated with poor performance on cognitive tasks and negative symptomatology [55].

Further support for DA dysfunction in the PFC comes from studies that have evaluated cognitive function in carriers of the valine allele of catechol-*O*-methyltransferase (COMT), an enzyme involved in DA degradation. This type of gene polymorphism increases the activity (methylation of dopamine) of COMT fourfold compared to the methionine allele, presumably resulting in lower functional DA levels in the PFC of people who carry the *Val* allele. Subjects with valine gene polymorphism demonstrate worse performance on cognitive tests of PFC function compared to the methionine allele carriers [56–59].

Thus, all these observations point in the direction that there is reduced DA function in the DLPFC of schizophrenia patients, although the exact nature of the pathophysiology underlying this perturbation (D₁ receptor dysregulation, decreased presynaptic DA release, excessive degradation of DA, or secondary to some other factor) is yet to be identified.

In summary, the literature reviewed above suggests, in its simplest form, that schizophrenia is associated with a functional *increase* in subcortical dopamine transmission which correlates with psychotic symptoms and is evident at illness onset and in acute exacerbation but not in stable outpatients, as well as a functional *decrease* in dopamine transmission in the PFC that appears to underlie deficits in cognition and negative symptomatology.

11.2.2 Glutamate (NMDA Receptor Hypofunction) Hypothesis

The second dominant pathophysiological hypothesis of schizophrenia is the glutamatergic dysfunction hypothesis. Glutamate acts on two families of receptors: ionotropic

receptors, which are rapid excitatory on–off cation channels, and metabotropic receptors, which are G-protein coupled. The ionotropic receptors are further subdivided into the kainate receptor, the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, and the *N*-methyl-D-aspartate (NMDA) receptor. The NMDA receptor is equipped with an ion channel regulating the penetration of calcium and other cations into the neuron. PCP binds to a specific site in this channel, thereby blocking the function of the receptor. Glycine, a coagonist, binds to the strychnine-insensitive binding site and enables glutamate to open the ion channel.

The glutamatergic dysfunction hypothesis evolved from observations that phencyclidine (PCP), a noncompetitive antagonist at the NMDA receptor, could induce a psychotic state in normals that was accompanied by negative symptoms and cognitive deficits that were similar to those seen in patients with schizophrenia [60, 61]. Subanesthetic doses of another noncompetitive NMDA receptor antagonist, ketamine, have also been shown to faithfully induce the spectrum of psychopathology of schizophrenia when administered to normal control subjects [62, 63]. Jentsch and Roth [64] contend that long-term repeated exposure to PCP (as opposed to acute exposure) faithfully mimics the various abnormalities observed in patients with schizophrenia, including positive symptoms, negative symptoms, and cognitive dysfunction, as well as hypofrontality in cerebral blood flow in functional brain-imaging studies. Patients with schizophrenia were found to be more sensitive to the deleterious effects of ketamine and demonstrated a worsening of positive and negative symptoms and cognitive deficits [65–67]. These observations led to the hypothesis that NMDA receptor hypofunction might be involved in the pathophysiology of schizophrenia [62, 68–70]. Farber and colleagues have hypothesized that decreased functioning of NMDA receptors leads to reduced inhibitory output from γ -aminobutyric acid (GABA)–ergic interneurons and consequent increased cortical glutamatergic excitatory output to limbic regions leading to symptoms of schizophrenia. Consistent with the NMDA receptor hypofunction hypothesis is the observation that glutamatergic agonist drugs such as glycine and D-serine have shown promising results in treatment of patients with schizophrenia; these will be reviewed later. Lastly, postmortem studies have identified abnormalities of glutamate receptor density in the PFC, thalamus, and temporal lobe of patients with schizophrenia [71–73].

11.2.3 Integration of Dopamine and Glutamate Hypotheses

There is substantial evidence of reciprocal interactions between the glutamatergic and DA systems. Acute administration of ketamine to normal volunteers resulted in substantial increase in striatal DA release as measured by reduction in [^{11}C] raclopride binding using PET imaging [74–76]. Chronic NMDA receptor antagonist administration *increases* subcortical DA release, particularly in the nucleus accumbens, and *decreases* mesocortical DA transmission in animals [64]. Chronic administration of NMDA receptor antagonists also results in decreased expression of the DA D₁ receptor messenger RNA (mRNA) in the PFC of rats and monkeys [64, 77, 78]; as mentioned above, the D₁ receptor has been shown to be critical for working memory function [53].

Various possible disturbances in the corticostriatal–thalamic–cortical loops that connect the PFC to subcortical structures have been postulated to account for increased subcortical DA transmission in the context of reduced PFC activity and to

attempt to integrate the DA and glutamate dysfunction hypotheses (see [23] for a review and synthesis). Weinberger postulated in 1986 [79], based on the seminal work of Pycoc et al. [80], that dysregulation of subcortical DA function in schizophrenia may be secondary to a failure of the PFC to adequately inhibit subcortical transmission. Mesocortical DA input has the net effect of inhibiting subcortical DA release through feedback loops. This is mediated in part by DA stimulation of GABAergic interneurons in the cortex which inhibit excitatory glutamate corticostriatal input to ventral tegmental area (VTA) DA neurons. Reduced DA input to the PFC (or dysfunctional DA transmission in the PFC because of impaired D₁ receptor function) would result in increased excitatory glutamatergic output to VTA DA neurons and increased subcortical DA transmission.

A second mechanism proposed to account for increased subcortical DA transmission in the context of PFC dysfunction was proposed by Carlsson [81]. He described a model in which the PFC modulates subcortical DA transmission via an excitatory pathway (direct corticostriatal glutamatergic pathway) and an inhibitory pathway (indirect via intermediate midbrain GABAergic neurons), with the inhibitory pathway normally in slight dominance. Subtle NMDA receptor hypofunction would be predicted to result in reduced stimulation of the midbrain GABAergic interneurons (indirect pathway) which would normally inhibit the midbrain DA neurons. The release of VTA DA neurons from the normal inhibitory GABAergic input would result in increased subcortical DA transmission.

The third hypothesized mechanism of dysfunction in the corticostriatal–thalamic–cortical circuitry involves the GABAergic neurons in the PFC that exert tonic inhibitory control over excitatory glutamatergic projections to the VTA DA neurons. A primary deficiency in PFC GABAergic neurotransmission would release the glutamatergic corticostriatal neurons from inhibition and thereby directly stimulate VTA DA neurons into overactivity. Consistent with this proposed GABAergic functional deficiency are the results of several postmortem studies that demonstrate an alteration of GABAergic function in the PFC of patients with schizophrenia (reviewed in [23]).

The preceding brief conceptual overview of the neurochemical pathophysiology of schizophrenia highlights several possible strategies for targeting therapeutic interventions for this disorder. Psychotic symptoms, presumably secondary to increased subcortical DA transmission, could be addressed from several directions: by selective D₂ antagonism in limbic regions; by increasing DA transmission in the PFC (e.g., reducing the rate of DA degradation, increasing DA release, or blocking reuptake); by augmenting GABAergic action in the PFC; or by modulating NMDA receptor/glutamatergic function in the PFC. Negative symptoms and cognitive deficits may respond to interventions that increase PFC DA activity (by direct D₁ stimulation, reducing the rate of DA degradation, increasing DA release, or blocking reuptake). These strategies, of course, need not be mutually exclusive, and a combination of various interventions that target disturbed neural circuits at various points may provide *maximal* benefit to most patients and relief to those with treatment-resistant illness.

Finally, a disease as complex and clinically varied as schizophrenia is not likely to result from a simple dysregulation in one neurotransmitter system [82]. The variability in the exact nature and magnitude of the neural-based dysfunction in schizophrenia and its interaction with normal maturational/developmental brain processes [48], coupled with the variability inherent in the dynamic interaction of an

individual with his or her environment, are likely to collectively contribute to the clinical heterogeneity of the disorder across individuals and within the same individual in different stages (first episode vs. chronic) and phases (relapse vs. remission) of the illness. Whether schizophrenia is the result of a single insult that simultaneously disrupts the development and/or function of multiple neurotransmitter systems or neural circuits or the consequence of an insult that primarily disrupts one system with all other observed perturbations occurring secondarily as a compensatory response is not yet known. The precise answer to this question, however, may not be as pertinent to clinical progress as is a thorough understanding of normal brain circuitry and the homeostatic mechanisms that maintain stability among the various neural circuits which are the underpinnings of normal human emotion and cognition and how these homeostatic mechanisms are disrupted in schizophrenia. This knowledge will surely provide us with numerous opportunities to correct an imbalanced neural network(s) at multiple prospective loci irrespective of the exact nature and neuropathological location of the primary insult responsible for the disorder.

11.3 HYPOTHESIZED MECHANISMS OF ACTION OF ANTIPSYCHOTICS

We will begin with a review of the evidence in support of D₂ receptor antagonism of antipsychotics as the fundamental action that underlies their therapeutic effect on psychosis. We will then review the various theories that have been postulated to account for the atypical profile of SGAs and review the evidence for other receptor actions of antipsychotic drugs and how that may relate to their efficacy profiles.

11.3.1 D₂ Receptor Occupancy and Antipsychotic Effect

A consistent observation that has stood the test of time about antipsychotic drug treatment is that every clinically effective antipsychotic reduces DA transmission to some extent; drugs that do not have never been shown to have antipsychotic effect. More specifically, the primary receptor target implicated in antipsychotic effect, as well as the induction of EPS and prolactin elevation, is the D₂ receptor. Recent examples of non-D₂ antagonists that failed in clinical trials in schizophrenia are MDL-100907 [serotonin type 2A (5-HT_{2A}) antagonist] [83], L-745,870 (D₄ antagonist) [84], fananserin (5-HT_{2A} and D₄ antagonist) [85], and SCH-39166 and NNC-01-0687 (D₁ receptor antagonists) [86–88].

Dopamine receptors exist in two broad families, D₁-like (D₁ and D₅) and D₂-like (D_{2, long/short}, D₃, D₄). The D₁ receptor has widespread distribution and is the predominant DA receptor in the PFC, while the D₅ receptor is localized in specific corticolimbic regions. The D₂ receptor has both presynaptic (autoreceptor) and postsynaptic location, and its distribution is in mesostriatal–limbic regions; it also mediates prolactin release in the pituitary. The D₃ and D₄ receptors have low-density corticolimbic distribution, with the D₃ receptor additionally exhibiting presynaptic autoreceptor location (see [89] for review). Seeman [90] was the first to directly associate the therapeutic actions of antipsychotic drugs to the D₂ receptor. He demonstrated a strong correlation between in vitro affinities of antipsychotic drugs for the D₂ receptor and their clinical potencies [34], although at that time the various

subtypes of the D₂ family had not been identified. Recent studies, however, using cloned cell lines have confirmed that clinical antipsychotic potencies correlate generally with their affinities for the D₂ receptor expressed independently of D₃ and D₄ subtypes [90, 91].

The advent of functional brain-imaging techniques (SPECT and PET) in the late 1980s gave the opportunity to address in vivo the nature of the exact relationship between D₂ receptor occupancy and the three cardinal properties of FGAs: antipsychotic effect, EPS, and prolactin elevation. Farde et al. [92] using PET and [¹¹C] raclopride as the radiotracer, evaluated in vivo D₂ receptor occupancy by various FGAs and clozapine and found D₂ receptor occupancies in the range of 70–89% and 38–63% in FGA- and clozapine-treated patients, respectively. Patients who had acute EPSs had significantly higher D₂ receptor occupancies. Nordstrom et al. [93] confirmed that lower D₂ receptor occupancy was required for antipsychotic effect than for induction of EPS. Subsequently, Kapur et al. [94] confirmed in a low-dose haloperidol study in patients in first-episode schizophrenia that occupancy thresholds of 65, 72, and 78% were related to antipsychotic affect, prolactin elevation, and EPSs, respectively. These and other studies have found that there was a wide variation in D₂ receptor occupancy among patients on the same dose of drug and that the thresholds, although generally accurate, were not invariably reliable in predicting clinical events. For example, a few patients responded to FGAs at low D₂ occupancy, while other subjects with >65% D₂ occupancy did not exhibit a response [94–96]. Similarly, on occasion, occupancy >72% was not associated with prolactin elevation or occupancy >78% was not associated with EPS [94]. The D₂ receptor occupancy also did not correlate with improvement (or lack thereof) of negative symptoms or cognitive deficits. Despite these limitations of not accounting for *all* clinical scenarios observed in patients with schizophrenia, the importance of this work cannot be underestimated as these data directly link in vivo D₂ receptor occupancy to antipsychotic effect, prolactin elevation, and EPSs.

Another question that has recently been addressed in the literature is the time to onset of antipsychotic effect. It has been a long-held belief that onset of antipsychotic effect is delayed by one to two weeks after initiation of treatment, and “depolarization inactivation” of dopamine neurons which occurs over about a two-week time frame (see [97] for a review) has been proposed as the underlying mechanism responsible for this delay. Kuhar and Joyce [98] proposed that drug-induced changes in protein synthesis or degradation following changes in gene expression could also explain the delay. However, a recent article by Agid et al. [99] questions the very existence of the delay in onset of antipsychotic effect. They reported a meta-analysis of 42 double-blind comparator-controlled studies and found that antipsychotic effect was evident within the first week of treatment and improvement in the first two weeks was greater than during any subsequent two-week period. Kapur et al. [100] reported that onset of specific antipsychotic effect was evident within the first 24 h in a double-blind placebo-controlled study comparing intramuscular olanzapine to intramuscular haloperidol and that improvement in psychosis was independent of change in agitation and excitement. Abi-Dargham also observed rapid improvement in psychotic symptoms upon DA depletion with α -methyl-*para*-tyrosine [47]. These observations are intriguing and clearly warrant further investigation. If true, they again support a direct role for DA in the mediation of psychosis and antidopaminergic activity in antipsychotic effect.

With the introduction of the second-generation antipsychotics, functional brain-imaging techniques were used to address the question of the underlying mechanism of atypicality. That the level of D₂ receptor occupancy was important in atypicality had been suggested by the work of Farde in 1992 [92], in which clozapine demonstrated lower D₂ receptor occupancy as compared to FGAs.

Two major theories have been postulated to account for an atypical profile; relatively higher 5-HT_{2A} versus D₂ antagonism and relative ease of staying below the ~80% D₂ occupancy threshold for EPSs with SGAs because of their loose binding (lower affinity) to the D₂ receptor.

11.3.2 High 5-HT_{2A} versus D₂ affinity

Meltzer [101] (also see Chapter 12 by Meltzer) proposed that higher 5-HT_{2A} versus D₂ receptor affinity conferred an atypical profile. This was based on observations that a higher in vitro 5-HT_{2A} relative to D₂ affinity distinguished the atypical antipsychotic class. 5-HT_{2A} antagonism can increase DA transmission in the nigrostriatal pathway and thereby mitigate the risk of EPSs and may also contribute to improvement of negative symptoms and cognitive dysfunction by increasing DA release in the PFC [101]. However, there are critical limitations to the “serotonin/dopamine” theory of atypicality of SGAs (reviewed in [102]): many FGAs (chlorpromazine, loxapine) have high 5-HT_{2A} affinity [103] and do not have an atypical profile; amisulpiride has no appreciable affinity for the 5-HT_{2A} receptor, and aripiprazole has higher D₂ compared to 5-HT_{2A} affinity, and yet both have atypical profiles; risperidone and olanzapine demonstrate high 5-HT_{2A} receptor occupancy at doses that are not antipsychotic (therefore 5-HT_{2A} antagonism per se cannot account for antipsychotic effect), and as the dose of these drugs is increased beyond the usual therapeutic range, the risk of EPSs increases despite saturation of the 5-HT_{2A} receptor system; the relative ratios of 5-HT_{2A}/D₂ receptor affinities of the SGAs do not predict their clinical EPS liability, for example, risperidone has the highest and quetiapine the lowest 5-HT_{2A}/D₂ ratio, but most clinicians would concur (and the data show [11, 104, 105]) that the EPS liability of risperidone is greater than that of quetiapine. Additionally, at equivalent D₂ receptor occupancies, risperidone and haloperidol are associated with comparable EPS liability [106]. Thus, high 5-HT_{2A} affinity may contribute to modulating DA in the striatum and PFC, but high 5-HT_{2A} occupancy does not protect from EPSs if D₂ occupancy is greater than the EPS threshold. The 5-HT_{2A}/D₂ hypothesis therefore does not satisfactorily explain atypicality [107].

11.3.3 D₂ Occupancy Thresholds and Rapid Dissociation

The second major hypothesis put forth to account for atypicality is the loose binding, or low affinity, of SGAs for the D₂ receptor as compared to FGAs [108–110]. This builds on the observation by Farde and Nordstrom (see above) that higher D₂ receptor occupancy was observed with FGAs compared to clozapine and was correlated with increased risk of EPSs. PET imaging studies with SGAs have demonstrated that at clinically therapeutic doses these drugs occupy between 50 and 75% of D₂ receptors [92, 111–117]. On the other hand, FGAs occupy in excess of 75% of available D₂ receptors at therapeutic doses [92, 94]. Lower D₂ receptor

occupancy in and of itself would account for the lower EPS liability of SGAs if the proposed EPS threshold of $> \sim 80\%$ D_2 receptor occupancy is true. Kapur and Seeman [110], in a further elaboration of this concept, suggested that rapid dissociation of SGAs from the D_2 receptor is the fundamental underlying molecular property that accounts for atypicality. They propose that the most important aspect of the affinity of a drug for the D_2 receptor is not how rapidly it binds to a receptor but how rapidly it dissociates from that receptor. The rate at which a drug dissociates from the receptor, or its k_{off} , is the most important determinant of how the drug and DA compete for the receptor. In a unit of time, a drug with fast k_{off} goes on and off a receptor much more frequently than a drug with a slow k_{off} . The faster the k_{off} , the more quickly the drug responds to DA surges, allowing for a more physiological DA transmission [110]. They conclude that this relationship between fast k_{off} and low affinity is the critical underlying molecular feature that explains how low affinity at the D_2 receptor leads to the atypical antipsychotic profile.

Another feature of atypicality that is exhibited by all SGAs except risperidone and amisulpiride is minimal elevation of serum prolactin. However, prolactin elevation has been shown to occur with all SGAs, but for most, it is transient and rapidly returns to baseline several hours after the last dose [118]. This has also been attributed to rapid dissociation of SGAs (fast k_{off}) from D_2 receptors in the anterior pituitary. Risperidone-induced elevation of serum prolactin has been attributed, at least in part, to higher peripheral compared to central distribution of its active metabolite 9-OH risperidone, leading to excessive D_2 blockade in the anterior pituitary which lies outside the blood–brain barrier [102].

In terms of D_2 receptor occupancy profiles, clozapine and quetiapine differ from ziprasidone, risperidone, olanzapine, amisulpiride, and aripiprazole, as they never exceed approximately 60–70% D_2 receptor occupancy even at the highest therapeutic doses. Because these drugs rapidly dissociate from the D_2 receptor, the level of occupancy rapidly drops off after the last dose (300–600 mg/day quetiapine or 350 mg/day clozapine used in these studies) such that 12 h later it is only 20% for quetiapine and 55% for clozapine [113, 115, 119]. A possible downside of rapid dissociation of clozapine and quetiapine from D_2 receptors could be the rapid emergence of psychotic symptoms upon drug discontinuation [120]. Although initially ziprasidone was found to have substantial D_2 receptor occupancy at low doses (20- and 40-mg single doses) in healthy volunteers [121], a recent study [122] found that 60% D_2 occupancy was not achieved until plasma levels corresponding to a 120-mg/day dose were reached, suggesting the optimal dose of this drug is 120 mg/day or higher. The predicted dose based on PET imaging of ≥ 120 mg/day is consistent with the clinical trial data for this drug [123–125]. Risperidone and olanzapine demonstrate increasing D_2 occupancy with increasing dose and also are associated with dose-related risk of EPSs [126]. The only SGA that does not appear to fit into the D_2 threshold schema is aripiprazole, a mixed DA partial agonist/antagonist [127]. In a study of 15 healthy men [128] who were treated with aripiprazole for a duration of two weeks, it was found that a dose of 2 mg/day of aripiprazole occupied between 70 and 80% of the striatal D_2 -like DA receptors. When the dose was increased to 30 mg/day, the receptor occupancy increased to almost 95% in the putamen, yet no associated EPS was evident as would be predicted for all other antipsychotics based on the $> 80\%$ D_2 occupancy threshold. The lack of EPS with aripiprazole has been attributed to its agonist activity at D_2

receptors. Similarly, the lack of efficacy in the presence of adequate D₂ receptor occupancy at the 2 mg/day dose is also not consistent with the 65% threshold hypothesis of efficacy [127] and could be accounted for by the partial agonist activity of aripiprazole at the D-2 receptor.

Thus both the 5-HT_{2A}/D₂ and D₂ receptor occupancy threshold/rapid dissociation theories have limitations as to their ability to account for *all* aspects of the disease or clinical profiles of *all* antipsychotics. These limitations, however, do not diminish the tremendous contribution made by these hypotheses to our understanding of antipsychotic drug action and drug development.

11.3.4 Highly Selective D₂/D₃ Antagonism, D₄ Antagonism, and Regional Specificity

Amisulpiride is a unique antipsychotic in that it is a highly selective, low-affinity antagonist at D₂/D₃ receptors. It has no activity at the D₁ or any other receptor. Low doses of amisulpiride (50–100 mg/day) occupy only 4–26% of striatal D₂ receptors [129] and seem to improve negative symptoms [130], while higher doses of 200–800 mg/day occupy 38–76% of D₂ receptors [129] and are effective against both positive and negative symptoms [131, 132]. It has been hypothesized that at low doses amisulpiride preferentially antagonizes presynaptic D₂/D₃ receptors, causing an increase in DA transmission in the PFC accounting for its efficacy in negative symptoms [133].

Another property of some antipsychotics that has been proposed to account for an atypical profile is D₄ receptor antagonism. The D₄ hypothesis grew out of observations of the receptor binding profile of clozapine (D₄ affinity > D₂) and the localization of this receptor in corticolimbic regions. However, this hypothesis has not withstood the test of time, and a study of a D₄ receptor antagonists L-745,870 not only did not demonstrate antipsychotic efficacy or EPS but was associated with worsening compared to placebo [84]. Sonepiprazole, a selective D₄ antagonist, yielded a negative outcome in a large double-blind, placebo-controlled randomized trial in 467 inpatients with schizophrenia in acute relapse [134]. Fananserine, a mixed 5-HT_{2A} and D₄ antagonist, was also found to be without antipsychotic effect [85]. Also, quetiapine and amisulpiride do not have D₄ affinity but have an atypical profile [135, 136].

Regionally specific preferential binding of SGAs to DA tracts projecting to the limbic region (as opposed to motor striatal projections) has also been proposed as a mechanism contributing to an atypical profile. Numerous studies in animals have found a selective effect of SGAs on A10 versus A9 DA neurons and inducing early gene expression in the nucleus accumbens and medial striatum as opposed to the dorsolateral striatum [137–140]. In humans, several SPECT and PET studies have demonstrated this regional specificity for clozapine, risperidone, olanzapine, amisulpiride, quetiapine, and sertindole [112, 141–146], although other studies using more rigorous analytical methods [52] have found no regional specificity for clozapine or risperidone [147, 148]. Further evaluation of this attribute is required to confirm its existence in humans and identify its underpinnings; its significance is evident as it raises the possibility of spatial targeting of drugs in the future.

11.3.5 Role of D₁ Receptors and Other Mechanisms

Activity at other receptors has also been postulated to mediate antipsychotic or other effects of antipsychotics, such as improvement in mood, cognition, and negative

symptoms. As described in Section 11.2.1, the D_1 receptor in the PFC is critically important in working memory function. Long-term FGA treatment induced working memory deficits in nonhuman primates that were reversed by brief treatment with a full D_1 agonist, and the beneficial effects were maintained over an extended period of time [54]. As working memory deficits have been consistently documented in patients with schizophrenia [149], drugs that have D_1 agonist activity might be particularly useful to target such a deficiency. No currently available antipsychotic has D_1 agonist activity.

Preclinical studies suggest that 5-HT_{2A} antagonism in the presence of D_2 antagonism results in increased DA release in the PFC, an effect that might be mediated by 5-HT_{1A} agonism [150]. Aripiprazole, clozapine, ziprasidone, and quetiapine are all partial agonists at the 5-HT_{1A} receptor. Clozapine, olanzapine, risperidone, and ziprasidone, but not haloperidol, increased prefrontal DA release [151–153]. Irrespective of the exact mechanism, increased prefrontal DA release in the PFC with the SGAs might contribute to improvement in negative symptoms, depressive symptoms, and cognitive dysfunction. Most SGAs, but not the FGAs, also increase release of acetylcholine in the PFC, which might lead to improved cognitive function [154]. Ziprasidone is also a norepinephrine and 5-HT reuptake inhibitor, and since the norepinephrine transporter is also functional in DA reuptake in the PFC, it is possible that this is another mechanism by which ziprasidone may increase DA transmission in the PFC. Zotepine also inhibits norepinephrine reuptake.

Lastly, some of the SGAs, but not the FGAs, have been shown to modulate NMDA receptor function at the cellular and behavioral levels in preclinical animal models [155–159]. Clozapine and olanzapine, but not haloperidol or raclopride, inhibit the electrophysiological effects of PCP in brain slices [158, 160, 161] and attenuate NMDA receptor antagonist-induced deficits in prepulse inhibition [155, 162]. As these drugs do not directly interact with the glutamatergic system, it is not clearly understood how they might be exerting their influence. One possibility for clozapine was recently highlighted in the report by Sur et al. [163] which showed that the active metabolite of clozapine, *N*-desmethylozapine, can potentiate hippocampal NMDA currents by its M_1 agonist activity.

11.3.6 Synthesis

From the above review several preliminary conclusions can be drawn about the mechanisms of action of antipsychotic drugs and the basis of atypicality: (1) D_2 receptor antagonism is the fundamental property of antipsychotic drugs that underlies their antipsychotic effect; (2) continuous D_2 receptor antagonism is not required for antipsychotic effect, but how intermittent this antagonism can be without losing antipsychotic efficacy is not known; (3) different thresholds of D_2 receptor occupancy are predictive of antipsychotic effect, prolactin elevation, and EPS liability; (4) D_2 antagonism alone does not explain antipsychotic effect across all patients, that is, patients may fail to respond despite adequate D_2 blockade; (5) 5-HT_{2A} receptor antagonism and activity at other 5-HT receptors (e.g., 5-HT_{1A}) contributes to clinical profiles of most but not all SGAs (amisulpiride is an exception); (6) activity at receptors other than or in addition to DA receptors will probably be required for amelioration of negative symptoms, depressive symptoms, and cognitive dysfunction as well as for patients with treatment-resistant illness; and (7) there is substantial

variability among the SGAs in receptor binding and clinical profiles—they are not members of one class.

Lastly, on a cautionary note, although remarkable advances have been made in the neurosciences over the past two decades, it is important to guard against overinterpretation of findings about the mechanisms that underlie the antipsychotic effect. There are limitations to our current conceptualizations of the receptor or intracellular basis of efficacy and side effects of antipsychotics. Studies done at a cellular level clearly do not reflect what happens in intact neural circuits of living animals. As an illustration, the output of a neuron in an intact neural circuit in response to an input may be influenced by its basal activity state and what other stimulatory or inhibitory inputs it receives at that time—the same input may result in opposite outputs depending on these and other factors. Not surprisingly, conflicting or unexpected results are not uncommon when ideas generated from basic science are taken to behavioral animal models and then to human disease states. We also know that the same receptor type may mediate completely different actions at different locations in the brain, for example, the D_2 receptor mediating antipsychotic effect, EPSs, and influence over prolactin secretion. What makes these receptors behave differently in different brain regions is not exactly known, and will this knowledge, once it accumulates, be able to guide us in developing strategies for spatial targeting of drugs? Additionally, although the relationships between degree of receptor occupancy *in vivo* and clinical effect has received recent attention, the temporal component of this interaction has not been rigorously evaluated. Is it better (or worse) to occupy 50–60% of D_2 receptors for 20% of the time (quetiapine) or 60% of the receptors for 100% of the time (or any combination thereof)? As noted earlier, an occasional patient may improve at low D_2 receptor occupancies. A study by Nyberg et al. [164] found that D_2 receptor occupancy for patients taking monthly injections of haloperidol decanoate dropped from 73% (60–82%) at week 1 postinjection to 52% (20–74%) at week 4. Also, the 25-mg biweekly dose of the long-acting injectable formulation of risperidone demonstrated robust efficacy [165] but was associated with only 25–48% D_2 receptor occupancy at trough plasma levels at steady state [166]. Similarly, in the long-term outpatient trial of Fleischacker et al. [167], 25 mg biweekly was found to be an effective dose. These observations are compatible with several mutually nonexclusive scenarios: (a) that a substantially lower level of D_2 occupancy than the proposed ~65% threshold may be compatible with antipsychotic effect (at least in some patients); (b) that, similar to the profile of clozapine and quetiapine, D_2 receptor occupancy with long-acting injectables exceeds the antipsychotic threshold only transiently, although with these formulations the time frame during which the antipsychotic threshold is exceeded is in days (not hours) followed by several days of “subtherapeutic” D_2 receptor occupancy; and, lastly, (c) that a lower level of D_2 receptor occupancy may be sufficient for maintenance of antipsychotic effect as compared to the 60–70% postulated for treatment of acute exacerbation. The last scenario is consistent with the finding by Laruelle et al. [168] that stable patients (as opposed to those in acute relapse) did not exhibit abnormal DA release upon an amphetamine challenge; yet we know that patients remain vulnerable to relapse secondary to perturbation of the DA system in response to stress. These and other relevant clinical questions will need to be addressed over the coming years.

11.4 CLINICAL PROFILES OF ANTIPSYCHOTIC DRUGS

11.4.1 First-Generation or Conventional Antipsychotics

The FGAs (phenothiazines, butyrophenones, thioxanthines) had been the mainstay of treatment for psychotic disorders from the early 1950s until the introduction of the first-line SGAs in the early 1990s. Their primary efficacy domain is in psychotic symptoms and they demonstrate minimal efficacy against negative symptoms and cognitive dysfunction and may actually worsen depressive symptomatology [169, 170]. About a third of patients in acute exacerbation have minimal response to these medications, and another 50% have a partial response [171, 172]. There are no significant efficacy differences among FGAs (reviewed in [173]). Until recently, the only long-acting injectable formulations available were of the FGAs; risperidone is now available in a long-acting injectable form (Risperdal Consta). The FGAs were routinely used in doses that were excessive leading to high rates of movement disorders, secondary negative symptoms, and further cognitive impairment because of anticholinergic effect of the drug itself or the anticholinergics prescribed to mitigate EPSs. Their side-effect profile included acute EPSs, tardive dyskinesia (TD), neuroleptic malignant syndrome, sedation, anticholinergic side effects, weight gain, and prolactin-induced side effects. The rates of acute EPSs in first-episode patients at therapeutic doses were as high as 70% [174], while TD risk was approximately 5% per year of exposure in the not elderly [175] and 25–30% per year in the elderly [176]. The high risk of TD, a potentially irreversible movement disorder, and the high rates of acute EPSs were the major motivating factors for psychiatrists to switch patients to SGAs when they became available beginning in the early 1990s.

11.4.2 Second-Generation or Atypical Antipsychotics

There is a wealth of double-blind placebo- and active comparator-controlled studies of the SGAs, most of them conducted to obtain regulatory approval. Such studies, among others, have been included in several recently published meta-analyses [11–13, 177] of the efficacy and safety/tolerability of SGAs compared to FGAs and SGAs compared to each other. In general, these studies did not include treatment-resistant patients, were conducted mostly in patients in acute exacerbation, were of relatively short duration, and assessed limited efficacy and safety outcome measures. Also, since most of the studies in these meta-analyses were sponsored by manufacturers of the new agents, it can be safely stated that the designs were at best neutral and at worst stacked to favor the new agent. Usually these design flaws were reflected in a higher than necessary dose of the comparator FGA, which would be expected to negatively influence dropout rates (because of increased risk of side effects), negative and depressive symptom ratings (because of EPSs that may mimic negative/depressive symptoms), and cognitive function (because of higher use of adjunctive anticholinergic medication to ameliorate EPSs).

The first meta-analysis by Leucht [11] included studies on risperidone, olanzapine, quetiapine, and sertindole. They found a very modest efficacy advantage on total symptomatology for risperidone and olanzapine compared to haloperidol (effect size r values of 0.06 and 0.07, respectively), with no difference for quetiapine and sertindole. On negative symptoms both olanzapine and risperidone were superior

to haloperidol, but again the effect size was very small. Sertindole was no different from haloperidol in the treatment of negative symptoms, and quetiapine in one study [178] was equivalent to chlorpromazine while in another [105] it was actually statistically *inferior* to haloperidol. Compared to haloperidol, all SGAs were associated with lower anticholinergic use, although the effect was weakest for risperidone. Interestingly, in a double-blind sertindole study that used a low 4-mg/day dose of haloperidol there was still a statistically higher use of anticholinergic medication in this group of patients compared to pooled sertindole dose groups, suggesting that even at relatively low doses high-potency FGAs are associated with significant EPS liability [11]. In two studies that used either a midpotency or low-potency FGA comparator (perphenazine, chlorpromazine), no difference in concomitant anticholinergic use was found compared to the SGA group. Because of lack of efficacy, dropout rates were statistically lower only for olanzapine compared to haloperidol; olanzapine also demonstrated lower dropout rates because of adverse events. Quetiapine had lower dropout rates compared to haloperidol related to adverse events and did not differ in dropouts for lack of efficacy; risperidone did not show an advantage over haloperidol in dropout rates related to either lack of efficacy or adverse events. This meta-analysis therefore suggested minor efficacy advantages for olanzapine and risperidone, moderate overall tolerability advantage for quetiapine and olanzapine, and a lower EPS liability as reflected in lower anticholinergic use for all the SGAs compared to haloperidol. However, in a second meta-analysis by Leucht [177] comparing SGAs to only low-potency FGAs, no advantage on EPS liability was seen for the SGAs (other than clozapine), although a moderate superiority in efficacy was documented for the SGAs.

The meta-analysis by Geddes et al. [12] was even less optimistic. They included 52 randomized trials with a total of 12,649 patients treated with risperidone, clozapine, olanzapine, quetiapine, amisulpiride, and sertindole. Overall, they found that SGAs had slightly superior efficacy and better tolerability and a lower risk of causing EPSs. However, when they controlled for comparator dose of the FGAs and separately analyzed data for patients who received 12 mg/day of haloperidol equivalents or less, all advantages of SGAs except for a modest EPS advantage disappeared. Essentially the conclusion from this meta-analysis was that other than a slight superiority on EPS profile, all other differences in outcomes between SGAs and FGAs could be accounted for by a higher than necessary dose of the comparator FGA.

The last meta-analysis conducted by Davis et al. [13] included 124 randomized trials of 10 SGAs versus FGAs and 18 trials comparing different SGAs. They found significantly greater effect size for efficacy for clozapine (0.49), risperidone (0.25), olanzapine (0.21), and amisulpiride (0.29). The mean effect sizes for the above drugs (other than clozapine) corresponded to about a four- to six-point advantage on the PANSS compared to FGAs. For perspective, the difference between these SGAs and the FGA comparator group was about half as much further improvement over that seen with FGAs compared to placebo. The effect size of efficacy of clozapine versus FGAs was double that of other SGAs versus FGAs. Quetiapine, sertindole, ziprasidone, aripiprazole, and remoxipride demonstrated similar efficacy as FGAs. Significance for superior efficacy for zotepine depended on the statistical method used and was significant with one method and just missed significance with another. Distinct from the results of the Geddes meta-analysis, Davis et al. did not find that the dose of the comparator FGAs had any effect on the outcome of their

analysis. A sub-meta-analysis of studies comparing SGAs to each other did not reveal statistical differences among them on efficacy profiles. However, clozapine was superior to risperidone if studies that used a low dose of clozapine were excluded from the analysis. The Davis meta-analysis did not address safety and tolerability issues.

Thus, collectively these meta-analyses suggest an EPS advantage for the SGAs (although the dose of FGAs was higher than necessary in many studies and there were minor differences among the SGAs) and probable superior overall symptom efficacy for clozapine, risperidone, olanzapine, and amisulpiride over FGAs, with clozapine demonstrating the most robust difference.

The claimed superiority of SGAs over FGAs on negative symptoms is even more problematic to address, because some features of EPSs caused by FGAs (flat affect, bradykinesia—so-called *secondary* negative symptoms) may be impossible to distinguish from the *primary* negative symptoms of the disease [179–182]. Additionally, negative symptoms could also be secondary to positive symptoms, depressive symptoms, and environmental deprivation [183, 184]. Modest differences have been demonstrated for risperidone and olanzapine compared to FGAs [18, 19] on negative-symptom improvement using path-analytic methods that attempt to remove the influence of psychotic, depressive, and extrapyramidal symptoms on negative symptomatology. These results should not engender significant enthusiasm as the analyses were performed post hoc and the most effective dose of the SGA was chosen—the gain in negative-symptom improvement, most of it in secondary negative symptoms, is modest at best.

Similarly, the superiority of SGAs on cognitive function improvement relative to FGAs is not well established. Impairments in cognitive test performance in patients on FGAs may result from EPSs, anticholinergic effects, and sedative effects, on top of what is due to the underlying disease process [185–187]. The improvement seen in cognitive function with SGAs may at least in part be attributable to elimination or relative reduction of some of these deleterious effects of FGAs. Additionally, optimal dosing of FGAs is of critical importance in these studies as excessive dose may result in sedation and impairment of motor function secondary to EPSs that negatively impacts performance on timed tests. In general, the SGAs have demonstrated improvement in verbal fluency, digit symbol substitution, executive function, and fine motor control [5, 188], and a meta-analysis by Keefe et al. [189] found a significant overall advantage for SGAs on cognitive test performance. However, in a prospective two-year study Green et al. [190] found no advantage on cognitive functioning for risperidone (mean dose 6 mg/day) compared to haloperidol (mean dose 5 mg/day) in stable outpatients with schizophrenia. Similarly, a recent double-blind randomized 12-week trial [191] comparing olanzapine (mean dose 9.6 mg/day) with low-dose haloperidol (4.6 mg/day) found that both drugs improved verbal fluency, motor function, working memory, verbal memory, and vigilance to a similar degree in primary analysis. On secondary analysis, a minor advantage was noted for olanzapine, but statistical significance levels were not corrected for in multiple analyses that were conducted to yield this conclusion. Thus the differences in cognitive improvement with SGAs versus FGAs, if both are dosed appropriately, appear to be minor. Further research is needed to definitively address this issue.

The potential of some of the SGAs to delay relapse as compared to FGAs has also been evaluated. In a double-blind randomized trial comparing risperidone to

haloperidol and utilizing appropriate doses of both drugs, Csernansky et al. [192] found that risperidone was associated with a significantly lower risk for relapse by study endpoint (Kaplan-Meier estimates of relapse risk were 34 vs. 60%, respectively). Data are also available for olanzapine in maintaining long-term therapeutic effect [17]. The data from the extension phases of three double-blind studies comparing olanzapine to haloperidol were pooled to form one group for each drug. Olanzapine-treated subjects experienced less relapse ($p=0.034$), and the Kaplan-Meier estimated one-year risk of relapse was 19.7% with olanzapine and 28% with haloperidol. Finally, in a meta-analysis of relapse prevention studies comparing SGAs to FGAs conducted by Leucht [193], the rate of relapse and overall treatment failure were modestly but significantly lower with the newer drugs. Methodological limitations identified by the authors that need to be addressed in future relapse prevention studies included the choice of comparator FGA, use of appropriate doses, application of clinically relevant relapse criteria, and monitoring of adherence. Collectively, these data suggest that the SGAs are associated with a modest reduction in relapse risk, although methodological limitations of the studies conducted thus far dampen this conclusion.

The use of SGAs is now routine in the treatment of first-episode patients because of their lower EPS burden, slightly superior symptom domain efficacy, and probable superiority in relapse prevention. At a neurochemical level, SGAs, based on the evidence from animal literature, may have effects on the NMDA system that are different from those of FGAs (see Section 11.3), thus providing another avenue for assertion of therapeutic effect distinct from direct D_2 antagonism. If the pathophysiology of schizophrenia is progressive, modulation of the glutamatergic system by SGAs may lead to better long-term outcomes [194]. Two recent studies [16, 195] of olanzapine and risperidone in first-episode patients have demonstrated that in treatment of acute phase of the illness both drugs were comparable to haloperidol in efficacy. However, at the last observation point (12 weeks) in the olanzapine study, significantly more patients were still in treatment in the olanzapine arm versus the haloperidol arm (67 vs. 54%). Similarly, in the risperidone-versus-haloperidol trial, which used appropriate doses for both drugs (~ 3 mg/day), relapse rates were 42 and 55% for risperidone- and haloperidol-treated patients, respectively, over a median follow-up period of 206 days. Additionally, risperidone was associated with a significantly longer median time to relapse (466 days for risperidone- vs. 205 days for haloperidol-treated patients). The advantage for risperidone in reducing relapse risk was evident even though both groups had comparable symptom improvement. Both SGAs were superior to haloperidol in EPS liability; olanzapine caused more weight gain and risperidone more prolactin elevation compared to haloperidol. Weight gain was initially greater with risperidone compared to haloperidol, but at study endpoint there was no difference. These studies therefore support the use of SGAs in the first episode of the illness, not because of superior acute treatment efficacy but due to modestly better long-term relapse outcomes.

Another area of significant importance in schizophrenia treatment research is the study of the prodromal period preceding the onset of psychosis. The usual time from onset of frank psychotic symptoms to treatment initiation is one year, but when the prodromal period is taken into account, the average delay is three years [196]. There is widespread interest in evaluating the utility of various interventions in the

prodromal stage of schizophrenia before the onset of overt psychosis. The broad goals of these efforts are to develop systems for early recognition of psychosis in high-risk individuals and develop strategies for secondary prevention—specifically, does intervention during the prodromal period reduce the risk of developing overt psychotic symptomatology, and in those who do develop the illness, do these interventions alter the course or severity of the illness? Research in this area is in its early stages. McGorry et al. [197] reported on a randomized, controlled, nonblinded trial in 59 “ultra-high-risk” individuals at late prodromal stages close to psychosis onset. They compared low-dose risperidone treatment (mean dose 1.3 mg/day) combined with cognitive behavior therapy to routine clinical care over a six-month period followed by six months of needs-based care. Significantly fewer subjects in the risperidone group had transitioned into psychosis at the six-month time point, and in those subjects who had been compliant with risperidone, the benefits extended through the ensuing six-month extension period even though risperidone had been stopped. It is however not possible from this study to determine the relative contribution of risperidone or the cognitive behavior therapy to the reduced risk of transition into psychosis [197]. Early psychosis recognition and intervention programs are being developed in several countries and we await the results of this important work.

Lastly, in treatment-resistant schizophrenia the evidence in support of clozapine is most robust and it is the only antipsychotic specifically approved by the U.S. Food and Drug Administration (FDA) for this indication as well as for reduction of suicide risk. In a review of seven studies Chakos et al. [4] found clozapine to be superior in efficacy to FGAs, produced less EPSs, and was associated with better compliance. The double-blind, randomized, active comparator-controlled (olanzapine) study that led to the suicide risk reduction indication for clozapine [10] included 980 patients who were at relatively higher risk of suicide that were followed for up to two years. Approximately 25% of patients had treatment-resistant schizophrenia. Suicidal behavior was significantly less in patients treated with clozapine versus olanzapine as reflected by fewer clozapine-treated patients attempting suicide (34 vs. 55; $p=0.03$), requiring hospitalizations (82 vs. 107; $p=0.05$) or rescue interventions to prevent suicide (118 vs. 155; $p=0.01$), or requiring concomitant treatment with antidepressants (221 vs. 258; $p=0.01$) or anxiolytics or soporifics (301 vs. 331; $p=0.03$). Clozapine remains a uniquely effective drug, especially for treatment-resistant schizophrenia, and its importance to contemporary antipsychotic research is evident in the fact that all theories of the neurochemical underpinnings of atypicality evolved from observations and research on its biochemical and clinical profiles.

11.4.3 Safety and Tolerability

The biggest advance in side-effect profiles of SGAs is the lower risk of acute EPSs. Among the types of EPSs, acute dystonias are very rare with SGAs, while akathisia still occurs with all the newer agents. Within the group, clozapine and quetiapine have the lowest (almost absent risk) of parkinsonism, while amisulpiride, risperidone, and olanzapine are associated with dose-related parkinsonism. Ziprasidone and aripiprazole can also occasionally induce EPSs, although it is not clear if there is a dose relationship with these drugs. Lower rates of TD have also been reported

with risperidone compared to haloperidol (0.6 vs. 4.1%, respectively) in a one-year double-blind study [192] and for olanzapine compared to haloperidol (0.5 vs. 7.4%, respectively) [198]. Weight gain has emerged as a major problem with some of the SGAs [199]. Olanzapine and clozapine are most notorious in this respect, and in some patients, rapid and massive weight gain can occur. Quetiapine and risperidone are intermediate in weight gain liability, while ziprasidone and aripiprazole rarely cause significant weight gain [200]. Weight gain in patients with schizophrenia is especially problematic as these patients are usually overweight at baseline, have high rates of smoking, and are usually physically inactive. This combination of risk factors topped off by an increased risk of glucose dysregulation and hyperlipidemia reported to varying degrees with these agents [200] can create a potentially fatal constellation of risk factors for cardiovascular disease and stroke. These adverse effects in their entirety may well be worse than tardive dyskinesia, which was the major concern for FGAs [201]. The diabetes risk appears to be greatest for clozapine and olanzapine, less for risperidone and quetiapine, and lowest for ziprasidone and aripiprazole [200]. Risperidone causes sustained prolactin elevation, and therefore patients who previously developed prolactin-induced side effects (galactorrhea, gynecomastia, menstrual irregularities, and sexual dysfunction) on older agents should be tried on other SGAs. Ziprasidone is associated with QT prolongation, although the significance of this in patients with no other risk factors is not clear. Clozapine, in addition to agranulocytosis risk and other side effects mentioned previously, is associated with significant orthostasis, seizures (dose related), myocarditis, constipation (at times severe), and various other side effects, making it a challenging drug to use in clinical practice. Recently, the FDA issued a black-boxed warning of increased death rate in elderly patients with dementia who were prescribed SGAs. This was based on a review of data from 17 placebo-controlled trials of SGAs (risperidone, olanzapine, aripiprazole, quetiapine) involving 5106 elderly patients with dementia-related psychosis. The results showed an approximate 1.6-fold increase in death rate with SGAs compared to placebo. The warning is applicable to all SGAs marketed in the United States. No controlled data are available for FGAs as regards to this risk. Additionally, from the same studies, an increased risk of stroke and other cerebrovascular adverse events were noted for risperidone, olanzapine, and aripiprazole (approximately 2–3 times greater rate of these events in drug vs. placebo). Data were not available for the other SGAs.

In summary, incremental gains have been made with the SGAs in terms of efficacy, but clozapine still remains unchallenged in its robust efficacy profile, especially in treatment-resistant patients. Significant reduction in the EPS burden with SGAs, however, is offset by problematic side effects of weight gain, diabetes, and hyperlipidemia.

11.5 DRUGS IN DEVELOPMENT AND FUTURE DIRECTIONS

This section will review drugs that are currently in clinical development or in preclinical evaluation as well as potential future interventional targets, some of which are relatively speculative at this time.

11.5.1 Drugs Acting Directly or Indirectly on Dopamine System

Asenapine is a 5-HT_{2A}/D₂ antagonist that is being jointly developed by Pfizer and Organon and is in phase III clinical trials. Early data from previous trials show good tolerability and superior efficacy when tested against a placebo. No information is available in the public domain about the nature or the methods of the study.

Bifeprunox is in phase III clinical trials. It is a partial DA agonist/antagonist as well as a 5-HT_{1A} receptor agonist. Early results report little to no weight gain and no cardiac or EPS effects. No further information is available.

Iloperidone is being developed by Titan Pharmaceuticals (currently in phase III FDA clinical trials) after being dropped by Novartis due to concerns that the drug may increase the QT interval. Iloperidone displays high affinity for norepinephrine α_1 adrenoceptors and DA D₃ and 5-HT_{2A} receptors and intermediate affinity for norepinephrine α_{2C} adrenoceptors and DA D₂ and D₄ and 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, and 5-HT₆ receptors [202]. This broad receptor profile makes it an interesting drug, although it has been in development for more than 10 years. In patients with schizophrenia treated with iloperidone, a low incidence of EPSs and weight gain has been shown. Data from phase II trials demonstrated efficacy in patients at doses of 8 mg/day and tolerability was good up to 32 mg/day [203].

Paliperidone extended release is the active metabolite (9-OH risperidone) of risperidone. It is a 5-HT_{2A}/D₂ antagonist and is being developed by Johnson and Johnson. No specific research information was found on this compound.

Ocaperidone has approximately equivalent antagonism of 5-HT_{2A} and D₂ DA receptors. It is nearing the end of phase II clinical trials.

Modafinil, a drug currently used to treat narcolepsy, is now being examined for its potential to improve cognitive symptoms and working memory in schizophrenia patients. The mechanism of action is still not entirely clear, although it appears that modafinil induces wakefulness through activation of sleep/wake centers in the hypothalamus and increases DA levels in the PFC. Turner [204] studied 20 chronic schizophrenic patients in a double-blind, randomized, placebo-controlled crossover study using a 200-mg dose of modafinil. Improvement was seen on short-term verbal memory span, with trends toward improved visual memory and spatial planning. This was accompanied by reduced response latency on the spatial planning task. Significant improvement in attentional set shifting was seen in schizophrenic patients, despite no effect of modafinil being seen in healthy volunteers or attention-deficit hyperactivity disorder (ADHD) patients on this task. Modafinil is currently in phase II clinical trials to test whether it improves working memory in schizophrenia patients with COMT gene variations.

Tolcapone is a COMT reversible inhibitor that acts in the PFC of the brain. By inhibiting the catabolism of DA it may be beneficial for the cognitive deficits associated with schizophrenia, especially working memory. Its measurable effects in rat brains include an increase in DA neurotransmitter levels in the PFC but not in the striatum [205], and it has been shown to improve cognitive dysfunction in advanced Parkinson's disease [206]. It can cause severe liver dysfunction and has been removed from the market in several countries. It is currently being evaluated in a National Institute of Mental Health (NIMH) placebo-controlled trial in patients with schizophrenia and normal controls with and without the high-risk COMT alleles.

11.5.2 D₄ Antagonists

The negative studies for the highly selective D₄ antagonist L-745,870 [84], the 5-HT_{2A}/D₄ antagonist finanserin [85], and sonepiprazole [134] were described earlier. The D₄ story does not look optimistic, at least in monotherapy.

11.5.3 D₃ Antagonist

The D₃ receptor has corticolimbic distribution and is of interest because most antipsychotics have high affinity for this receptor [207]. In a postmortem study of schizophrenia, D₃ receptors were increased in the limbic striatum of drug-free patients, while it was normal in those treated with antipsychotics [208]. D₃ antagonists have been developed (S33084, SB-277011-A, AVE5997), but there are only limited animal behavioral data at this time. Controlled trials of D₃ antagonists will help clarify the differential contributions of the D₂ and D₃ receptor in the mediation of antipsychotic effect.

11.5.4 D₁ Agonists and Antagonists

The relevance of this receptor to cognitive function was covered earlier in the chapter. In the early 1990s there was interest in D₁ receptor antagonists as possible antipsychotic agents because they were active in preclinical models predictive of antipsychotic effect [91]. However, clinical trials of SCH-39166 [86, 87] and NNC-01-0687 [88] demonstrated no antipsychotic activity. On the other hand, there is substantial interest in D₁ agonists for their potential role in cognitive enhancement, and positive results have been obtained in nonhuman primates [54]. DAR 0100 (dihydroxidine) is a high-affinity short-acting D₁ agonist that has been studied in Parkinson's disease [209] and is currently being tested as a treatment for cognitive deficits in schizophrenia.

11.5.5 Neurotensin Agonist/Antagonist

Neurotensin, an endogenous neuropeptide, is colocalized with DA neurons that project specifically to the PFC and nucleus accumbens and it acts as a modulator of DA function [210]. Neurotensin was proposed to have potential antipsychotic activity more than two decades ago [211] (see [212] for a review). The problem with developing neurotensin agonist/antagonists is their rapid degradation by peptidases and difficulty crossing the blood–brain barrier. Centrally administered neurotensin demonstrates behavioral and biochemical effects very similar to antipsychotics [213]. A recent placebo- and haloperidol-controlled randomized trial to evaluate the utility of four potential novel agents in the treatment of schizophrenia included the neurotensin (NTS1) antagonist SR 48692 as one of the agents [214]. It did not demonstrate any therapeutic effect. However, it has been proposed that neurotensin *agonists* may be more relevant to the treatment of schizophrenia [215]. Richelson et al. [215] have developed a neurotensin agonist, (2*s*)-2-amino-3-(1*H*-4-indoyl) propanoic acid, that is resistant to peptidases and enters the brain if injected outside the brain. It is currently in preclinical toxicology testing.

11.5.6 Neurokinin Antagonists

Neurokinin 3 (NK3) receptors appear to regulate midbrain DA activity [216, 217]. A NK3 antagonist in development as potential treatment for schizophrenia is osanetant [218]. In the study of four novel compounds referred to above, Meltzer et al. [214] found that patients treated with SR 142801 demonstrated significant global and psychotic symptom improvement, though not of the same magnitude seen in the haloperidol group.

11.5.7 Drugs Acting on Glutamate System

If NMDA receptor hypofunction contributes to the pathophysiology of schizophrenia, then drugs that enhance NMDA receptor function could potentially be useful in the treatment of this disorder. Various strategies are being employed to modulate the glutamatergic system. Glycine is an obligatory coagonist at the NMDA receptor, and this site represents an interesting target for NMDA receptor augmentation. Studies with glycine have yielded mixed results with very high dose studies showing some benefit (see [20] for a review). This probably results from poor brain penetration of glycine. More optimistic results have been obtained with the glycine site agonists D-cycloserine and D-serine when they are added to ongoing antipsychotic treatment (except when added to clozapine). Patients demonstrated improvement in negative and cognitive symptoms, but less so on positive and depressive symptomatology. D-Serine appears to be the most promising as it has better permeability at the blood–brain barrier than glycine and does not have the partial agonist properties of D-cycloserine. In an eight-week add-on trial of D-serine in treatment-resistant patients with schizophrenia, D-serine treatment was associated with improvement in negative, cognitive, and positive symptomatology [219]. In the first systematic review and meta-analysis of glutamatergic drugs for schizophrenia, Tuominen [220] found a beneficial treatment effect on negative symptoms of schizophrenia only for add-on treatment with glycine and D-serine. The average improvement on the PANSS-negative subscale was a modest four points. There was also a trend toward positive effect on cognitive symptoms for glycine and D-serine, although further trials are needed to confirm this finding [220]. In this review D-cycloserine was not found to be helpful in schizophrenia and may even have been detrimental.

Another approach to augmentation of glycine at the NMDA receptor is to inhibit its reuptake, thereby increasing intrasynaptic concentration of glycine. A couple of glycine transporter inhibitors (sarcosine, glycyldodecylamide) show some potential in animal models for antipsychotic effect [221–224]. In a recent six-week double-blind, placebo-controlled add-on trial of sarcosine for the treatment of schizophrenia, significant improvement in positive, negative, and cognitive symptoms was evident in the sarcosine group [225].

Glutamate reuptake inhibitors can control glutamatergic transmission by removal of glutamate from the synapse. Postmortem studies in schizophrenia have revealed alterations in gene expression of glutamate transporters [226, 227]. Theoretically glutamate reuptake inhibitors could have potential utility in schizophrenia, although no such agent is currently in testing.

There is also interest in group II metabotropic glutamate receptors (mGluR2/3) which are presynaptically located and may function as autoreceptors regulating

glutamate release [228, 229]. Inconsistent results in behavioral animal models have been obtained thus far with mGluR2/3 agonists ([229]). However, in a preliminary human trial in normal subjects, the mGluR2/3 agonist LY354740 attenuated ketamine-induced working memory impairment [230].

The non-NMDA ionotropic glutamate receptors, AMPA, and kainate receptors could potentially mediate some of the behavioral effects of increased glutamate release seen following NMDA antagonism with drugs such as ketamine. Antagonists at these receptors have been shown to reverse some of the deficits induced by NMDA antagonism in pharmacological and behavioral animal models [231–234]. These data suggest that AMPA/kainate receptor antagonists could potentially have utility for the treatment of cognitive deficits [231]. In contrast to the agents discussed above, ampakines *augment* AMPA receptor function and enhance long-term potentiation, learning, and memory in rodents [235, 236]. It is suggested [20] that ampakines, by potentiating AMPA receptor-induced depolarization, indirectly enhance NMDA receptor function. CX516, the first member of this class, synergistically blocked methamphetamine-induced rearing behavior in rats when it was added to clozapine and to conventional antipsychotic agents, an effect believed to predict antipsychotic efficacy [237]. In preliminary human trials in patients with schizophrenia, CX516 improved cognitive and negative symptoms in patients who were also taking clozapine [238]. However, in a recent double-blind, placebo-controlled small study of patients with schizophrenia who were partially refractory to treatment with FGAs, CX516 in monotherapy did not produce impressive effects on positive and cognitive symptoms [239].

Memantine is a noncompetitive NMDA receptor antagonist. Although speculative, it is possible that memantine, by antagonizing NMDA receptors on GABAergic interneurons in the PFC, could potentially reduce the GABAergic (inhibitory) input to the glutamatergic neurons resulting in an increased glutamatergic output. Forrest Pharmaceuticals is currently sponsoring a phase II double-blind, placebo-controlled efficacy and safety trial of memantine added on to atypical antipsychotics in patients with schizophrenia.

Lamotrigine added to ongoing antipsychotic treatment has yielded some positive outcomes. Kremer et al. [240] conducted a 10-week, double-blind, placebo-controlled study in 38 treatment-resistant schizophrenia inpatients receiving conventional and atypical antipsychotics. Patients were randomized in a 2 : 1 ratio to receive adjuvant treatment with lamotrigine, gradually titrated to a 400-mg/day dose, or placebo. Of these, 31 completed the trial. In primary last observation carried forward analysis, no statistically significant between-group differences were observed; however, completer analyses revealed that lamotrigine treatment resulted in significant reductions in positive and general psychopathology symptoms; no significant differences in lamotrigine effects were noted between conventional versus atypical antipsychotics. In a double-blind, placebo-controlled add-on crossover trial involving 34 hospitalized treatment-resistant patients on clozapine Tiihonen [241] found that 200 mg/day lamotrigine was effective in reducing positive and general psychopathological symptoms, whereas no improvement was observed in negative symptoms. The positive impact of lamotrigine on psychotic symptoms may be mediated by DA modulation. Lamotrigine has been demonstrated to reduce DA levels in the striatum of mice presumably by inhibition of tyrosine hydroxylase, thereby reducing DA synthesis [242]. Lamotrigine also reduces glutamate release and in a controlled study

of normal volunteers was shown to reduce the extent of neuropsychiatric disruptions in response to ketamine [243]. Lamotrigine is now in phase III clinical trials as an adjunctive treatment for schizophrenia.

11.5.8 Noradrenergic Agents

Norepinephrine plays an important role in cognition in the PFC mediated by α_2 receptors [244–248]. The α_2 agonist clonidine has been shown to improve PFC-mediated cognitive function in patients with schizophrenia [249]. Guanfacine, a selective α_2 agonist, when added to risperidone or FGAs in a four-week placebo-controlled trial, resulted in significant improvement in tasks of working memory and attention, but only in the risperidone-treated subjects [250]. Thus, α_2 agonism may mediate improvement in some aspects of the cognitive function in schizophrenia. On the other hand, clozapine and risperidone are potent α_2 antagonists. Litman et al. [251, 252] reported that the addition of idazoxan, an α_2 antagonist to FGAs produced a “clozapine-like” profile. Antagonism at the α_2 receptors therefore, may contribute to the “antipsychotic” efficacy profile of antipsychotics.

11.5.9 Cholinergic Agents

The α_7 nicotinic acetylcholine receptor (nAChR) modulates auditory gating and has been shown to be reduced in certain brain regions of patients with schizophrenia in postmortem studies, and genetic studies link the α_7 nAChR gene to sensory processing deficits in schizophrenia (reviewed in [253]). Agonists at the α_7 nAChR are in development for clinical trials in schizophrenia.

The $\alpha_4\text{-}\beta_2$ nAChR represents more than 90% of the high-affinity nicotinic binding sites in the rat brain and is believed to play an important role in the actions of nicotine. SIB-1553A is an agonist at this receptor that appears to enhance performance in spatial and nonspatial working memory and reference memory in aged rodents and monkeys [254, 255]. These observations make $\alpha_4\text{-}\beta_2$ nAChR agonists interesting candidates for evaluation in the treatment of cognitive dysfunction in schizophrenia.

Galantamine is a positive allosteric modulator of nAChRs and also an acetylcholinesterase inhibitor. The allosteric interaction essentially amplifies the action of acetylcholine at pre- and postsynaptic nicotinic receptors. Presynaptic nAChRs are capable of modulating the release of acetylcholine, glutamate, GABA, and 5-HT. Case reports suggest that adjuvant galantamine improves negative symptoms in schizophrenia [256, 257]. An exploratory phase II study of galantamine in patients with schizophrenia who were smokers was recently completed in Canada. The results are not yet known.

A recent double-blind trial of donepezil, a reversible acetylcholinesterase inhibitor, did not show any positive effect when added to risperidone on cognitive function associated with schizophrenia [258].

Lastly, it is well known that cholinergic muscarinic receptors modulate dopaminergic and glutamatergic neurons (reviewed in [259]). Recently the *N*-desmethyl metabolite of clozapine was found to have potent partial agonist activity at this receptor, and it was demonstrated that it could potentiate NMDA currents in the hippocampus [163]. Examples of M_1 agonists include xanomeline and the M_2/M_4

agonists PTAC and BuTAC (reviewed in [259]). Xanomeline has been shown to have positive effects on cognitive and psychotic symptoms in Alzheimer's disease patients. Accumulating data suggest that muscarinic partial agonists might be efficacious in treating not only positive but also negative and cognitive symptoms of schizophrenia.

11.5.10 Other Agents

Acute cannabis intoxication can produce schizophrenia-like symptoms [260] and long-term use may induce "negative" symptoms [261]. These and other observations have led to a cannabinoid hypothesis of schizophrenia [262]. A cannabinoid CB1 antagonist (SR141716) recently failed to reveal any therapeutic effects in the four-novel-compound double-blind, placebo- and haloperidol-controlled six-week study of Meltzer et al. [214].

Other attempts at increasing dopamine in the PFC have included double-blind augmentation trials of the monoamine oxidase type B (MAO_B) inhibitor selegiline [263] and DA reuptake blocker mazindol [264], both of which did not yield a positive outcome on the primary outcome measure of improvement of negative symptoms.

Neurosteroids such as dehydroepiandrosterone (DHEA) and its sulfate derivative (DHEA-S) have demonstrated positive outcomes in case reports [265–267] and one double-blind study [268] as an adjunct to antipsychotic treatment, with improvement in negative, depressive, and anxiety symptoms, especially in women. Further studies with these and other neurosteroids are ongoing.

11.6 CONCLUSION

The last 15 years have been a period of rapid advance in our knowledge about the pathophysiology of schizophrenia, the mechanisms of actions of our drugs, and the number of potential therapies that are in development. An important area that was not covered here is pharmacogenomics (see [269, 270]) in which the relationships between specific genetic polymorphisms and the pharmacokinetic, pharmacodynamic, and clinical profiles of a drug are identified in populations of patients. This line of research will allow a more precise matching of patients with the drugs that they are more likely to respond to and/or better tolerate. At this time, we do not have reliable objective predictors of drug response or side-effect vulnerability.

Schizophrenia is a disease of multiple symptom domains and tremendous inter individual variability in clinical presentation and course. Currently we treat patients with one type of medication which is effective primarily against psychotic symptoms. As we get effective treatments for negative symptoms and cognitive dysfunction, it will likely be routine for a patient to be treated with a combination of drugs individually targeting the specific domains of psychopathology in that individual. The treatments for acute-phase illness will likely be different from maintenance strategies, either qualitatively or quantitatively.

Lastly, we are already moving beyond receptor pharmacology to the exploration of intracellular mechanisms of signal transduction, signaling pathways, and the factors that influence gene expression. Eventually, a thorough understanding of the factors that underlie cell development, plasticity, and resilience may enable us to develop entirely new classes of pharmacotherapeutic agents that exert their effects

intracellularly, and we may ultimately be able to stop deterioration and even reverse cumulative morbidity in schizophrenia, the most disabling of mental illnesses.

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12

ATYPICAL ANTIPSYCHOTIC DRUGS: MECHANISM OF ACTION

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12.1	Introduction: Distinction Between Typical and Atypical Antipsychotic Drugs	411
12.2	Clozapine and Related Drugs: Dopamine D ₂ Receptor Blockade	412
12.3	D ₁ , D ₃ , and D ₄ Receptors: Substituted Benzamides	415
12.4	Partial Dopamine Agonists	416
12.5	Role of 5-HT _{2A} , 5-HT _{2C} , 5-HT _{1A} , and Other 5-HT Receptors	417
12.6	Atypical Antipsychotics and 5-HT _{2A} Receptor	418
12.7	5-HT _{2A} Receptor Blockade, Enhancement of Cortical DA Efflux, and Cognitive Function in Schizophrenia	421
12.8	5-HT _{2A} Receptor Blockade and Extrapyramidal Function	422
12.9	Role of 5-HT _{2C} Receptor in Antipsychotic Drug Action: 5-HT _{2A} and 5-HT _{2C} Interactions	422
12.10	Role of 5-HT _{1A} Receptor in Antipsychotic Drug Action: 5-HT _{1A} and 5-HT _{2A} Interactions	424
12.11	Role of 5-HT ₆ Receptor	425
12.12	Role of Serotonin Release in Antipsychotic Drug Action	426
12.13	α_2 - and α_1 -Adrenergic Mechanisms and Atypical APDs	426
12.14	Glutamatergic Mechanisms	427
12.15	Cholinergic Mechanisms	429
12.16	Neurokinin 3 Receptors	430
12.17	Neurogenesis	430
12.18	Conclusions	431
	Acknowledgments	431
	References	431

12.1 INTRODUCTION: DISTINCTION BETWEEN TYPICAL AND ATYPICAL ANTIPSYCHOTIC DRUGS

Antipsychotic drugs (APDs), by definition, suppress hallucinations and delusions, which are core features of schizophrenia as well as other neuropsychiatric disorders with psychotic features. Those APDs which produce catalepsy in rodents and extrapyramidal side effects (EPSs) in humans within the same dose range (e.g.,

chlorpromazine and haloperidol) are called typical APDs or neuroleptics. Acute EPSs (parkinsonism, dystonias, neuroleptic malignant syndrome) and delayed EPSs (tardive dyskinesia, tardive dystonia) represent disturbing and sometimes life-threatening side effects which interfere greatly with the patient's quality of life and compliance with treatment. Clozapine, discovered five years after chlorpromazine, was the first APD which did not produce catalepsy and virtually no EPSs. Because of this dissociation between antipsychotic efficacy and EPSs, clozapine was called an *atypical* APD. This name has persisted, although some clinical investigators incorrectly refer to typical APDs as first-generation antipsychotics and to clozapine and all subsequent atypical APDs as second-generation antipsychotics, even though many typical APDs which are still in use were discovered and introduced 5–10 years after clozapine (e.g., molindone and thiothixene) [1].

As will be discussed, there are important clinical differences between typical and atypical APDs beyond EPS liability. Clozapine (but not all other atypical APDs) was found to be effective to treat delusions and hallucinations in 60–70% of the 30% of patients with schizophrenia who have persistent psychotic symptoms despite treatment with typical APDs [2, 3] or with other atypical APDs [4]. Clozapine produces agranulocytosis in about 1% of patients [5]. For this reason, it has generally been used only in patients who do not respond to or cannot tolerate other APDs [3]. Atypical APDs are also able to modestly improve negative symptoms compared with typical APDs [6]. The advantage for efficacy for some atypical APDs over typical APDs has recently been challenged in a large, pragmatic clinical trial that had fewer constraints on patient population than most previous studies [7]. Most importantly, they have been reported to improve some domains of cognition in patients with schizophrenia [8, 9]. However, the advantages for cognition are modest, not apparent in all domains, and further research is needed to establish definitively that they do have an advantage over typical neuroleptic drugs to improve cognition. The advantage of greater efficacy for these components of schizophrenia coupled with advantages for EPS, both acute and chronic (e.g., tardive dyskinesia), has led to this group of compounds becoming the most widely prescribed APDs in all countries where they are freely available.

There are now numerous types of atypical APDs. As indicated in Table 12.1, there are at least seven subtypes of atypical APDs which have been identified, although not all are clinically available and, indeed, the efficacy of some of these agents is still not adequately established. In addition, there are many other proposed atypical APD mechanisms which cannot be reviewed here for space limitations.

As can be seen in Table 12.1, there is a diversity of mechanisms which are now believed to be able to achieve some degree of control of psychosis with significant sparing of EPSs. This review will consider current information on the mechanism of action of these drugs, concentrating on clozapine and related drugs because most is known about its actions.

12.2 CLOZAPINE AND RELATED DRUGS: DOPAMINE D₂ RECEPTOR BLOCKADE

There are numerous theories of why clozapine is atypical, that is, produces low EPSs at clinically effective doses. Historically, the low EPSs due to clozapine were

TABLE 12.1 Atypical Antipsychotic Drugs**Clozapine-like serotonin (5-HT_{2A})/D₂****Receptor antagonists/inverse agonists**

Asenapine
 Clozapine
 Lurasidone
 Melperone
N-Desmethylozapine
 Olanzapine
 Paliperidone
 Perospirone
 Quetiapine
 Risperidone
 Sertindole
 Ziprasdione
 Zotepine

Partial dopamine agonists

Ariprazole
 Bifeprunox

5-HT_{2A/2C} antagonists

SR43469B
 ACP 103
 M100907

D₂ antagonists/5-HT_{1A} agonists

SLV313
 SSR181507
 S16924

Substituted benzamides

Amisulpride
 Remoxipride

Muscarinic agonists

Xanomeline
N-Desmethylozapine

Neurokinin 3 antagonists

Osanetant
 Talnetant

attributed to its relatively weak affinity for the D₂ receptor, its antimuscarinic properties, its lack of effect on the firing of nigro striatal dopamine (DA) neurons compared to ventral tegmental (VTA) DA neurons, or to D₁ receptor antagonism (see [10, 11] for a review). The typical APDs have been suggested to diminish psychotic symptoms by blockade of D₂ receptors in mesolimbic nuclei, especially the nucleus accumbens (NAC), stria terminalis, and extended amygdala, while blockade of D₂ receptors in the caudate/putamen (dorsal striatum) has been shown to be the

major basis for parkinsonism and other EPSs (see [10] for review). For the typical APDs, there is a very high correlation between the estimated therapeutic dose of these drugs and their affinity for the D₂ receptor [12, 13]. Actions of these agents at other receptors (e.g., M₁ muscarinic receptors, histamine H₁ receptors, and α_1 and α_2 adrenoceptors) have been thought to contribute to side effects such as drowsiness, weight gain, and constipation rather than efficacy.

Positron emission tomography (PET) studies utilizing ¹¹C-raclopride as the ligand have shown that occupancy of 65–80% of striatal D₂ receptors is required for an antipsychotic action by typical APDs and risperidone or olanzapine and that $\geq 80\%$ occupancy is associated with EPSs (see [14, 15] for a review). However, clozapine and quetiapine are effective at lower occupancies of D₂ receptors than typical and other atypical APDs listed with it in Table 12.1 (e.g., risperidone, olanzapine, and ziprasidone), implying some mechanism other than D₂ receptor blockade is contributing to their low-side-effect profile and perhaps their efficacy as well [14, 16–19]. However, caution is needed in interpreting these studies because they do not assess the occupancy of VTA, ventral striatal, or cortical D₂ receptors, all of which are more likely to be related to antipsychotic action than is the dorsal striatum, which is the region of interest in studies using ¹¹C-raclopride in the first generation of PET studies. This ligand is much less useful than high-affinity substituted benzamides which have been developed more recently. Kessler et al. [20] performed [¹⁸F]fallypride PET studies in six schizophrenic subjects treated with olanzapine and six treated with haloperidol to examine the occupancy of striatal and extrastriatal D₂/D₃ receptors. [¹⁸F]setoperone PET studies were performed in seven olanzapine-treated subjects to determine 5-HT_{2A} receptor occupancy. Occupancy of DA D₂ receptors by olanzapine was not significantly different from that seen with haloperidol in the putamen, ventral striatum, medial thalamus, amygdala, or temporal cortex, that is, 67.5–78.2% occupancy; no preferential occupancy of DA D₂ receptors was seen in the ventral striatum, medial thalamus, amygdala, or temporal cortex. There was significantly lower occupancy of substantia nigra (SN)/VTA D₂ receptors in olanzapine- than haloperidol-treated subjects, that is, 40.2 versus 59.3% ($p = 0.01$); in olanzapine-treated subjects, the SN/VTA DA D₂ receptor occupancy was significantly lower than that seen in the putamen, that is, 40.2 versus 69.2% ($p = 0.01$). Sparing of SN/VTA DA D₂ receptor occupancy was suggested to contribute to the low incidence of EPSs in the olanzapine-treated patients. Similar results have now been obtained with clozapine- and quetiapine-treated patients [21]. Clozapine, the most effective of these agents, was found to produce the lowest occupancy of SN/VTA D₂ receptors. It is not possible to resolve the SN and VTA binding. Thus, it could be that binding by the atypical APDs to either or both regions is reduced. In any case, it does not seem likely that this alone could account for all the differences between typical and atypical APDs. Grunder et al. [22] studied the occupancy of extrastriatal D₂/D₃ DA receptors by clozapine using [¹⁸F]fallypride in 15 patients with schizophrenia. Mean D₂/D₃ receptor occupancy was statistically significantly higher in cortical (inferior temporal cortex 55%) than in striatal regions (putamen 36%, caudate 43%, $p < 0.005$). Occupancy of cortical receptors approached 60% with plasma clozapine levels in the range of 350–400 ng/mL, which corresponds to the threshold for antipsychotic efficacy of clozapine. These authors concluded that extrastriatal D₂/D₃ receptor binding of clozapine may be more relevant to its antipsychotic actions than striatal binding.

It has been suggested that atypicality may be due to rapid dissociation from the DA D₂ receptor due to its relatively easy displacement by surges of endogenous DA [23, 24]. It has also been proposed that rapid and extensive displacement of clozapine and quetiapine from binding sites by endogenous DA accounts for the low occupancy of striatal D₂ receptors by these drugs [23]. However, it is not clear on what basis these agents achieve their antipsychotic action if they are equally easily displaced from limbic D₂ receptors. Moreover, numerous atypical APDs (e.g., sertindole, risperidone, asenapine, and ziprasidone) are comparable to haloperidol in their rate of dissociation from the D₂ receptor, based upon their equally high affinity for that receptor. Thus, this theory fails to account for the atypical profile of these high-D₂-affinity atypical APDs. However, relatively easy displacement from D₂ receptors of low-D₂-affinity atypical APDs such as clozapine and quetiapine could contribute to their very low risk of EPSs and greater tolerability in patients with Parkinson's disease. Seeman [25] has proposed the fast-off theory as the basis for the atypical features of amisulpride, but it is difficult to understand what distinguishes it from other D₂/D₃ antagonists which are not atypical. The fast-off theory has had no apparent impact on the process of antipsychotic drug discovery development and, thus, has not met that important clinical test. This is in marked contrast to the hypothesis, to be discussed, that atypicality is related to the combination of a partial decrease in dopaminergic function coupled to blockade of 5-HT_{2A} receptors or stimulation of 5-HT_{1A} receptors [26, 27].

12.3 D₁, D₃, AND D₄ RECEPTORS: SUBSTITUTED BENZAMIDES

The central nervous system (CNS) contains four subtypes of DA receptor other than the D₂ receptor, the D₁, D₃, D₄, and D₅ receptors. Whether these DA receptors play a role in the action of current antipsychotic drugs or are potential targets for future ones is poorly understood. Phenothiazine and thiothanthines, but not butyrophenones such as haloperidol or substituted benzamides such as amisulpride, are effective D₁ antagonists. Clozapine is a D₁ partial agonist [28] and has a greater extent of occupancy of D₁ receptors than any other antipsychotic drug [16]. There is some indirect evidence that the ability of the atypical APDs to improve cognition in schizophrenia may be mediated through effects on D₁ receptors via their role in long-term potentiation [29]. Specific D₁ receptor antagonists have been evaluated in the treatment of schizophrenia, but no clear evidence of beneficial therapeutic activity was obtained in pilot studies [30, 31].

The cloning of the D₄ receptor provoked much interest in the possibility that this was a key mechanism in the action of atypical APDs. This idea first originated from the observation that clozapine differed from other antipsychotics in having significantly higher affinity for the D₄ than for the D₂ receptor [32], a finding not supported by a number of other studies. Interest in the D₄ receptor as a target for antipsychotic drugs has waned after the completion of three negative randomized clinical trials with D₄ receptor antagonists fananserin [33], L-745,870 [34], and sonepiprazole [35].

Schwartz et al. [36] have suggested that the D₃ receptor, which is relatively highly expressed in limbic and cortical areas, may represent an important target for antipsychotic drugs which generally have similar affinities for D₂ and D₃ receptors. Clozapine has modest affinity for D₃ receptors although most of the other atypical

APDs related to clozapine do not. The discriminative stimulus for clozapine sometimes but not always generalizes to D_3 antagonists [37]. SB-277011-A has high affinity and selectivity for the D_3 receptor and good brain bioavailability [38]. This compound was active in preventing isolation-induced deficits in prepulse inhibition (PPI), a putative animal model for antipsychotic activity, but was not effective to block either amphetamine- or phencyclidine (PCP)-induced locomotor activity, which has much more validity in that regard [38]. However, subchronic administration of SB-277011-A selectively decreased the firing rate of VTA but not nigrostriatal DA neurons in the rat, indicating a clozapine-like profile [39]. In a microdialysis study in rats, the acute administration of SB-277011-A significantly increased extracellular levels of DA, norepinephrine (NE), and acetylcholine (ACh) without affecting levels of 5-HT in the anterior cingulate region of the medial PFC (mPFC). As will be discussed, this is similar to the profile we and others have reported with clozapine and other atypical APDs [40, 41]. Clinical trials of selective D_3 antagonists have not yet been reported.

The substituted benzamides are an interesting group of APDs and like the butyrophenones may include both typical and atypical APDs. The prototype is sulpiride, which is generally thought of as a typical APD, while the drug in widespread use in Europe but not the United States is amisulpride [42]. It has been suggested that the low EPS profile of sulpiride and amisulpride is due to selective antagonism of D_2/D_3 receptors in the mesocortico/limbic system [43]. Using single-proton emission computerized tomography (SPECT) with [^{123}I] epidepride as the ligand, eight amisulpride-treated patients (mean dose 406 mg/day) showed moderate levels of D_2/D_3 receptor occupancy in the striatum (56%) and significantly higher levels in the thalamus (78%) and temporal cortex (82% [44]). This finding suggests that modest striatal D_2 receptor occupancy and preferential occupancy of limbic cortical dopamine D_2/D_3 receptors may be major factors which explain the therapeutic efficacy and low-EPS profile of amisulpride. However, the risk of tardive dyskinesia with sulpiride is higher than that with other typical APDs [45]. On the other hand, the risk with amisulpride has been reported to be in the same range as other atypical APDs [46]. However, no differences in the mechanism of action of these two drugs have been reported as yet, suggesting there is some additional feature of the pharmacology of amisulpride that has yet to be identified. Another contrast is that sulpiride has been reported to impair cognitive functioning in normal volunteers [47] while amisulpride has been reported to improve some aspects of cognition in schizophrenia, but the data are very limited and did not involve a control group [48]. The clinical profile of amisulpride and its mechanism of action are worthy of more extensive investigation.

12.4 PARTIAL DOPAMINE AGONISTS

Most antipsychotic drugs which have affinity for D_2 and D_3 receptors are inverse agonists at both receptors [49]. However, some are partial D_2/D_3 agonists, for example, aripiprazole, bifeprunox [50], and *N*-desmethylozapine (NDMC), the major metabolite of clozapine [49, 51, 52]. Partial agonists, by definition, produce lesser activation of the D_2/D_3 receptors than the full agonist and as such will produce smaller functional responses than the endogenous neurotransmitter [53]. Partial

agonists are particularly more potent as agonists when there is high receptor reserve. Thus, DA partial agonists are more likely to stimulate DA autoreceptors than postsynaptic DA receptors. Because of this, they diminish dopaminergic stimulation through a dual mechanism: suppression of the release of DA by stimulating autoreceptors and blocking the response to the full agonist at postsynaptic receptors. There have been many attempts to develop DA autoreceptor agonists for the treatment of schizophrenia but all failed until recently, when DA partial agonists, which also had 5-HT_{2A} inverse agonist or 5-HT_{1A} partial agonist properties or both, were tested and were found to be effective and tolerable. Thus aripiprazole, an approved atypical antipsychotic drug [51], is a D₂ partial agonist and both a 5-HT_{2A} inverse agonist and 5-HT_{1A} partial agonist. Bifeprunox is a partial agonist at both the D₂ and 5-HT_{1A} receptors only, with no 5-HT_{2A} antagonist properties [50]. It is in phase III testing as an antipsychotic drug. Both drugs produce minimal EPSs at clinically effective doses. As agonists, both suppress serum prolactin levels. Aripiprazole, but not bifeprunox, has been reported to reverse deficits in social interaction in rats produced by the NMDA receptor antagonist PCP [52]. The contribution of 5-HT_{2A} inverse agonism or 5-HT_{1A} partial agonism to their efficacy will be discussed subsequently. Functional selectivity of some DA agonists, including partial agonists, for specific types of DA receptors (e.g., autoreceptors vs. postsynaptic D₂ receptors) [54, 55] has been demonstrated, that is, differential activation of different isoforms of a D₂ receptor, thus implying variable “intrinsic activity” of an agonist [56]. Whether aripiprazole or bifeprunox also demonstrate functional selectivity for D₂ or D₃ receptors and whether this contributes to their atypical antipsychotic properties remain to be seen. Li et al. [57] have reported that aripiprazole, at a dose of 0.3 mg/kg, which corresponds to clinically effective doses, can increase the release of DA in the rat mPFC and hippocampus. These results have been replicated [58]. It will be of interest to determine if there are any partial DA agonists, with no significant affinity for 5-HT_{2A} or 5-HT_{1A} receptor which are effective and tolerable APDs or whether some other potentiating mechanism (e.g., M₁ agonism, to be discussed) is needed.

12.5 ROLE OF 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1A}, AND OTHER 5-HT RECEPTORS

Clozapine and a group of other atypical antipsychotic drugs have a greater affinity for 5-HT_{2A} than D₂ receptors [26, 59, 60]. The hypothesis that a relatively high affinity for the 5-HT_{2A} receptor compared to their affinities for the D₂ receptor was the basis for the difference between atypical and typical APDs contributed to the development of the newer antipsychotic agents listed above, all of which support the previously mentioned hypothesis of high affinity for 5-HT_{2A} and low affinity for D₂ receptors [59, 60]. However, other 5-HT receptors may be important in the action of clozapine and other recently introduced antipsychotic agents or of potential value for developing more effective or better tolerated antipsychotic agents. These include 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₆, and 5-HT₇ receptors [61]. While some of the atypical APDs developed on the basis of the 5-HT_{2A}/D₂ hypothesis also have affinities for 5-HT_{2C}, 5-HT₃, 5-HT₆ or 5-HT₇ receptors in the same range as that for the 5-HT_{2A} receptor, this is not characteristic of all of these agents and, thus, it is not likely that affinities for these receptors are primary factors contributing to the

low-EPS profile of the entire class of agents [60, 62, 63]. However, this does not rule out that actions at various 5-HT receptor contribute to low-EPS effects of specific drugs or other actions, for example, cognitive improvement or improvement in negative symptoms.

12.6 ATYPICAL ANTIPSYCHOTICS AND 5-HT_{2A} RECEPTOR

5-HT_{2A} receptors have been implicated in the genesis of as well as the treatment of psychosis, negative symptoms, mood disturbance, and EPSs. The hallucinogenic effect of indole hallucinogens has been related to stimulation of 5-HT_{2A} rather than 5-HT_{2C} receptors [64]. Numerous studies have examined the density of 5-HT_{2A} receptors in various cortical regions of patients with schizophrenia with decreased [65, 66], increased [67], or normal levels reported. It is well established that some typical and atypical APDs can decrease the density of 5-HT_{2A} receptors [68] so the postmortem results noted above may be related to drug treatment. A PET study in never-medicated patients with schizophrenia confirmed this decrease in cortical 5-HT_{2A} receptor density [69], but other studies which were not exclusively with never-medicated patients found no decrease in 5-HT_{2A} receptors in the cortex of patients with schizophrenia [70, 71]. Further study of this important issue is warranted. The antipsychotic effect of clozapine and other atypical ADPs has been attributed, in part, to its ability to block excessive 5-HT_{2A} receptor stimulation without excessive blockade of D₂ receptors [26]. This conclusion is consistent with the high occupancy of 5-HT_{2A} receptors produced by clozapine at clinically effective doses and its low occupancy of D₂ receptors (in the 30–50% range as measured with the [³H]raclopride), the latter being significantly below the 80–100% occupancy usually produced by typical neuroleptic drugs [16, 21, 72]. The occupancy of 5-HT_{2A} and D₂ receptors has been studied with other atypical APDs such as risperidone, olanzapine, sertindole, and quetiapine with results similar to those of clozapine; all are more potent 5-HT_{2A} inverse agonists and D₂ antagonists at appropriate doses, but less so than clozapine. Some of these agents (e.g., risperidone) produce high D₂ occupancy at moderately high doses [18, 72] but do not cause the level of EPSs associated with such high levels of occupancy when produced by typical APDs. The most likely reason for this difference is the blockade of 5-HT_{2A} receptors by the atypical APDs.

The bell-shaped dose–response curve of risperidone, with higher doses being less effective than lower doses [73], suggests that excessive D₂ receptor antagonism may diminish some of the beneficial effects of 5-HT_{2A} receptor blockade [63]. This is supported by two recent studies in which risperidone was added to clozapine treatment of partial responders in placebo-controlled randomized clinical trials. Placebo was generally superior to or equal to risperidone in a number of outcome measures [74, 75]. The highly selective 5-HT_{2A} inverse agonist M100907, formerly MDL 100907, has been found in a controlled study to have some efficacy for treating positive and negative symptoms in hospitalized schizophrenic patients [76]. However, because it was less effective than haloperidol, no further testing in schizophrenia followed. Nevertheless, the concept that 5-HT_{2A} receptor blockade may be useful to treat some forms of psychosis, especially when combined with weak D₂ receptor blockade, is deserving of further testing. The 5-HT_{2A/2C} selective antagonist SR46349B [77] was recently reported to be nearly as effective as haloperidol in

treating patients with schizophrenia in a double-blind clinical trial [78]. NRA0045, which has potent 5-HT_{2A}, D₄, and α_1 but negligible D₂ or D₃ receptor blockade, has been found to have atypical antipsychotic properties in rodents [79, 80]. The selective 5-HT_{2A/2C} inverse agonist ACP 103 [81, 82] is being tested as monotherapy in the treatment of 3,4-dihydroxy-L-phenylalanine (L-DOPA) psychosis and schizophrenia. Low doses of three atypical APDs, clozapine, quetiapine, and melperone [83], are effective and tolerable in the treatment of L-DOPA psychosis. We have proposed that this is due to their 5-HT_{2A} receptor blockade, sparing D₂ receptors [84]. The role of 5-HT_{2A} receptor blockade in the action of clozapine and possibly other drugs with potent 5-HT_{2A} affinities is supported by the evidence that the *His452Tyr* allele of the 5-HT_{2A} receptor, which is present in 10–12% of the population, is associated with a higher frequency of poor response to clozapine [85]. In addition, the T102C single-nucleotide polymorphism has been reported to be related to response to clozapine [86]. Taken together, the evidence from clinical trial data suggests that 5-HT_{2A} receptor blockade may contribute to antipsychotic drug action.

There is additional evidence consistent with the relevance of 5-HT_{2A} receptor blockade for APD action. Thus, M100907, or other selective 5-HT_{2A} receptor inverse agonist, either alone or in combination with selective antagonists of other receptors, has been found to be effective in various animal models of psychosis. These include (a) blockade of amphetamine-induced locomotor activity and inhibition of the firing of VTA dopaminergic neurons [87]; (b) blockade of PCP- and dizocilpin (MK-801)-induced locomotor activity [88, 89]; (c) blockade of MK-801-induced prepulse inhibition [90]; and (d) antipsychotic-like activity in the paw test [91], among others. Of particular interest is the report of Wadenberg et al. [92] that the combination of an ED 50 dose of raclopride, a D₂ receptor antagonist, and M100907, but not M100907 alone, was effective in blocking the conditioned avoidance response. They concluded that 5-HT_{2A} antagonism alone could not achieve an antipsychotic action but that minimal blockade of D₂ receptors was required to achieve such an effect. This corresponds much more closely to the apparent clinical situation than does the models above where M100907 alone was effective, for example, blockade of PCP- or MK-801-induced locomotor activity. ACP 103 is another selective 5-HT_{2A/2C} inverse agonist [93] which has been shown to potentiate the effect of haloperidol and risperidone to enhance cortical DA efflux in rats [94]. Gardell et al. [95] have recently reported that ACP 103 reduced the dose of haloperidol and risperidone required for activity in the amphetamine- or MK-801-induced hyperactivity models and suppressed haloperidol-induced hyperprolactinemia in mice.

Additional evidence for the greater importance of 5-HT_{2A} and D₂ receptor interactions in the regulation of mesocorticolimbic versus nigrostriatal DAergic activity comes from two studies of Olijslagers et al. [96, 97] using midbrain rat slices and electrophysiological recording of the activity of A9 and A10 DA neurons. They first reported that 5-HT (20 μ M) in combination with quinpirole (30 nM), a D₂ agonist, enhanced the reduction of the firing rate in both A9 and A10 DA neurons, compared to quinpirole (30 nM) alone. However, the 5-HT_{2A/2C} agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 500 nM) enhanced quinpirole-induced reduction of firing rate in the A10 but not A9 DA neurons. The selective 5-HT_{2A} receptor antagonist MDL100907 and the selective 5-HT_{2C} receptor antagonist SB242084 (50 and 500 nM) both abolished the enhancement of quinpirole-induced reduction by either 5-HT or DOI, suggesting the involvement of direct and

indirect (possibly via interneurons) modulation pathways in A10. 5-HT_{2A} receptors have been localized on the cell bodies of the A10 DA and other nondopaminergic VTA neurons [98].

Olanzapine and clozapine, two atypical APDs, more potently reversed the amphetamine-induced inhibition in A10 neurons compared to A9 neurons [96]. Olijslagers et al. [96] also reported that risperidone (0.03 and 0.1 μ M) reversed amphetamine-induced inhibition of firing activity similarly in A9 and A10. Risperidone has a higher affinity for D₂ relative to 5-HT_{2A} receptors than either clozapine or olanzapine. The dopamine D₂ receptor antagonist (–)sulpiride (0.05 and 1 μ M) reversed the amphetamine (10 μ M)–induced inhibition of firing activity in A9 and A10 neurons. However, the selective 5-HT_{2A} receptor antagonist M100907 (0.05 μ M), strongly enhanced the reversal of amphetamine-induced inhibition by (–)sulpiride in A10, but its effectiveness to enhance the effect of sulpiride was much less in A9 DA neurons. They suggested that 5-HT_{2A} in combination with DA D₂ receptor antagonism may play a role in atypical APD differential effects on nigrostriatal and mesocorticolimbic DAergic activity, leading to some of the clinical differences between typical and atypical APDs.

The 5-HT_{2A} and 5-HT_{1A} agonist psilocybin has been reported to impair cognitive function in normal volunteers [99]. Increased dopaminergic activity in the NAC and other mesolimbic and possibly cortical regions may contribute to positive symptoms, including formal thought disorder. The 5-HT_{2A/2C} agonist DOI, which is hallucinogenic in humans, itself had no effect on basal DA release, potentiated amphetamine-induced DA release, and attenuated the ability of apomorphine, a direct-acting D_{1/2/3} agonist, to decrease DA release in the striatum [100]. There is now considerable evidence from both behavioral and neurochemical studies involving *N*-methyl-D-aspartate (NMDA) antagonists such as PCP and MK-801 that 5-HT_{2A} receptors modulate activate but not basal mesolimbic DAergic function [88, 101, 102]. Thus, stimulated DA release (e.g., with stress) may be increased in the forebrain terminal regions secondary to enhanced stimulation of 5-HT_{2A} receptors. Agents which block the effect of excessive but not basal 5-HT_{2A} receptor stimulation may be the most useful clinically. M100907 has been found to diminish the increase in DA efflux in the NAC produced by haloperidol [103] or *S*-sulpiride [27]. M100907 infused directly into the mPFCs resulted in a concentration-dependent blockade of K(+)–stimulated DA release and also blocked increases in DA release produced by the systemic administration of DOI. Thus, local 5-HT_{2A} antagonism has an inhibitory effect on stimulated DA release and suggest that cortical 5-HT_{2A} receptors potentiate the phasic release of mesocortical DA [104]. The local (in the mPFC) and systemic administration of DOI increased the firing rate and burst firing of DA neurons and DA release in the VTA and mPFC [105]. The increase in VTA DA release was mimicked by the electrical stimulation of the mPFC. The effects of DOI were reversed by M100907. These results indicate that the activity of VTA DA neurons is under the excitatory control of mPFC 5-HT_{2A} receptors. Taken together, these data suggest that blockade of cortical 5-HT_{2A} receptors, by itself, may have antipsychotic action when dopaminergic activity is slightly to moderately increased. More studies are needed to define the ability of 5-HT_{2A} receptor antagonists to potentiate the action of low doses of D₂ receptor blockers in animal models as well as the clinic. Jakab and Goldman-Rakic [106] have demonstrated that 5-HT_{2A} receptors are located on most cortical pyramidal neurons, especially those above and below layer

IV, as well as on many (γ -aminobutyric acid) (GABA)–ergic interneurons known to specialize in the perisomatic inhibition of pyramidal cells: large- and medium-size parvalbumin- and calbindin-containing interneurons. Together they may play a crucial role in psychosis by virtue of their ability to modulate intracortical and cortical–subcortical glutamatergic neurotransmission. This could contribute to the ability of 5-HT_{2A} inverse agonists, including those atypical APDs which are potent in this regard, to attenuate some of the behavioral effects of PCP and ketamine.

12.7 5-HT_{2A} RECEPTOR BLOCKADE, ENHANCEMENT OF CORTICAL DA EFFLUX, AND COGNITIVE FUNCTION IN SCHIZOPHRENIA

As previously mentioned, clozapine and related atypical APDs (e.g., risperidone and olanzapine) have been shown to modestly improve selected areas of cognitive function in most patients with schizophrenia [9]. The effect of these agents on cognition may be dependent, in part, upon their ability to increase the release of DA in PFC and hippocampus, which depends, in part, on their serotonergic actions [107, 107a]. We have found that the combination of a saturating dose of a 5-HT_{2A} inverse agonist/antagonist and a dose of D₂ antagonist which only partially blocks D₂ receptors leads to increased DA release in the frontal cortex [94, 108, 109]. The 5-HT_{2A} receptor blockers do not potentiate D₂ receptor blockade when the dose of the latter is saturating [108]. This is consistent with the hypothesis that at clinical doses atypical APDs achieve partial blockade of D₂ receptors relative to 5-HT_{2A} receptor blockade. In addition, the atypical APDs enhance efflux of ACh in the PFC and hippocampus of rodents [110–113]. It is possible that this effect may also contribute to their ability to improve cognition.

5-HT_{2C}, 5-HT₃, 5-HT_{1A}, and 5-HT₄ receptors have also been reported to have significant effects on ACh release in the rat PFC [114–118]. Impairment of working memory in humans following administration of the 5-HT_{1A} agonist flesinoxan has been reported [119]. Sumiyoshi et al. [120], on the other hand, have found that tandospirone, a 5-HT_{1A} partial agonist, can improve other domains of cognition in patients with schizophrenia treated with typical neuroleptic drugs.

Brain-derived neurotrophic factor (BDNF) regulates survival, differentiation, synaptic strength, and neuronal morphology in the cerebral cortex and hippocampus in frontal and other cortical areas while decreasing its expression in the dentate gyrus granule cell layer. The BDNF gene *Val66Met* polymorphism has been reported to be associated with schizophrenia and response to clozapine [121]. It has recently been demonstrated that stimulation of 5-HT_{2A} receptors by the 5-HT_{2A/2C} agonist DOI and stress increases the expression of BDNF in cortex and hippocampus [122]. These effects were blocked by the 5-HT_{2A} antagonist M100907 and ritanserin, respectively, which by themselves did not have any effect in these regions. Electrophysiological studies with slices from rats following chronic treatment with clozapine or haloperidol showed that chronic clozapine but not haloperidol treatment resulted in an attenuation of the effect of the activation of 5-HT_{2A} receptors without changing response to 5-HT_{1A} and 5-HT₄ receptor activation. These data are consistent with the hypothesis that chronic clozapine selectively attenuates 5-HT-mediated excitation in neuronal circuitry of the frontal cortex while leaving 5-HT-mediated inhibition intact [123]. Clozapine and olanzapine upregulated BDNF messenger RNA (mRNA)

expression in CA1, CA3, and dentate gyrus regions of the rat hippocampus whereas haloperidol (1 mg/kg) downregulated BDNF mRNA expression in both CA1 ($p < 0.05$) and dentate gyrus ($p < 0.01$) regions [124]. Neither acute nor chronic clozapine treatment significantly affected the expression of BDNF mRNA in various brain areas. However, the NMDA antagonist MK-801 (5 mg/kg; 4 h) significantly increased BDNF mRNA in the entorhinal cortex, an effect which was attenuated by pre-treatment with clozapine and haloperidol [125]. These data suggest that clozapine-like atypical APDs, via their 5-HT_{2A} antagonism, might modulate the activity of BDNF and possibly other growth factors.

Transcription factors of the Nur family (Nurr1, Nur77, and Nor-1) are orphan nuclear receptors that have been associated with DA neurotransmission. Nurr1 is involved in midbrain DA neuron development. Nur77 and Nor-1 are expressed in dopaminergic areas such as the striatum, NAC, and PFC. Modulation of Nur77 and Nor-1 mRNA expression by antipsychotics can be used to calculate an index that is predictive of the typical or atypical profile of antipsychotic drugs [126]. Inductions of Nur by antipsychotic drugs was correlated with DA D₂ receptor in the striatum and NAC. The 5-HT_{2A}/D₂ affinity ratio of antipsychotics also predicted these patterns of inductions. These data suggest that typical and atypical APDs might induce distinct Nur-dependent transcriptional activities, which may contribute to their pharmacological effects.

12.8 5-HT_{2A} RECEPTOR BLOCKADE AND EXTRAPYRAMIDAL FUNCTION

Several lines of evidence suggest that potent 5-HT_{2A} receptor blockade is relevant to the low-EPS profile of clozapine but that 5-HT_{2A} receptor blockade by itself cannot explain the low-EPS liability of these agents. In order to test the role of 5-HT_{2A} receptor blockade, Meltzer et al. [26] studied a group of compounds which had antipsychotic activity in human or animal models that are thought to be predictive of antipsychotic activity and which produced less EPSs in humans or weak catalepsy in animals. The ratio of affinity for 5-HT_{2A} to D₂ receptors was the best predictor of atypicality [26]. This continues to be supported by further research [11]. Ishikane et al. [127] reported that M100907 is able to block haloperidol-induced catalepsy only at low doses of haloperidol. Weiner et al. [128] profiled antipsychotic drugs for functional activity at 33 of the 36 known human monoaminergic G-protein-coupled receptors using the mammalian cell-based functional assay Receptor Selection and Amplification Technology (R-SAT). Competitive antagonism of D₂ receptors and inverse agonism of 5-HT_{2A} receptors were nearly uniform throughout this class, with typical agents demonstrating low 5-HT_{2A}/D₂ ratios and atypical agents demonstrating high ratios.

12.9 ROLE OF 5-HT_{2C} RECEPTOR IN ANTIPSYCHOTIC DRUG ACTION: 5-HT_{2A} AND 5-HT_{2C} INTERACTIONS

There has been extensive consideration given to the role of 5-HT_{2C} receptors in the action of atypical APDs. The 5-HT_{2C} receptor is found throughout the CNS,

including the VTA and the NAC [129]. The 5-HT_{2C} receptor is constitutively active, meaning it is activated even in the absence of agonist [130, 131]. The *Ser23Cys* single-nucleotide polymorphism of the *HTR2C* receptor gene has been reported to be predictive of response to clozapine [132]. The *HTR2C* receptor gene undergoes extensive RNA editing in the brain, which leaves open the possibility that there are multiple forms of the receptor in brain [133]. This in turn suggests the *HTR2C* gene may be very important to epigenetic events which may influence the course of schizophrenia and response to treatment [134, 135]. Because of the development of specific 5-HT_{2C} agonists, inverse agonists, and antagonists, it has been possible to obtain evidence for a tonic inhibitory action of 5-HT_{2C} receptors on the burst firing of mesolimbic and mesocortical dopaminergic neurons. Thus, the firing rate of VTA DA neurons is inhibited or increased by 5-HT_{2C} agonists or antagonists, respectively. This is consistent with microdialysis studies which show that 5-HT_{2C} antagonists increase extracellular concentrations of DA in the NAC and mPFC [101, 136, 137]. These studies establish that the 5-HT_{2C} receptor is most important for regulation of tonic DA release. We have recently reported that the combination of the 5-HT_{2C} neutral antagonist SB242984 1.0 mg/kg and low- or high-dose haloperidol enhanced the release of cortical and NAC DA in rats using microdialysis [82]. SB242084 0.2 mg/kg produced significant increase in DA efflux in the NAC, not the mPFC. The extent of both 5-HT_{2C} and 5-HT_{2A} receptor blockade, in relation to D2 receptor blockade, may be a key factor in the relative ability of atypical APDs to preferentially increase cortical DA efflux compared to NAC DA efflux [82].

Early studies found no significant differences between groups of atypical APDs, and typical neuroleptics with regard to the affinity for 5-HT_{2C} receptor or the difference between 5-HT_{2C} and D₂ affinities have been reported [60, 62, 138]. A large group of atypical antipsychotic drugs (sertindole, clozapine, olanzapine, ziprasidone, risperidone, zotepine, tiospirone, fluperlapine, tenilapine) displayed potent inverse agonist activity at rat and human 5-HT_{2C} receptors [139]. Typical antipsychotic drugs of various chemical classes did not. Several typical antipsychotic drugs (chlorpromazine, thioridazine, spiperone, thiothixene) displayed neutral antagonist activity by reversing clozapine inverse agonism. These data are consistent with the concept that 5-HT_{2C} receptor blockade of constitutive and stimulated 5-HT_{2C} receptor stimulation may play a role in the action of atypical APDs and be involved in the etiology of schizophrenia and other forms of psychosis. Chronic sertindole but not clozapine downregulated cortical 5-HT_{2C} receptors [140]. 5-HT_{2C} receptor antagonism appears to have useful effects on certain types of memory impairment. Thus, SB-200646 antagonized memory impairment due to some 5-HT_{2A/AC} agonists and dizocilpine but not scopolamine [141]. Of the approved atypical APDs, some have equivalent affinities for the 5-HT_{2A} and 5-HT_{2C} receptors (clozapine, olanzapine, sertindole) while others are more selective for the 5-HT_{2A} receptor (risperidone, quetiapine, ziprasidone). This difference roughly corresponds with potential to produce weight gain in that clozapine and olanzapine cause the greatest weight gain, quetiapine intermediate, and aripiprazole, bifeprunox, risperidone, and ziprasidone the least. The 759T polymorphism in the promoter region of the 5-HT_{2C} receptor gene has been found to be associated with clozapine-induced weight gain, response to clozapine, and tardive dyskinesia [134].

An interesting aspect of the 5-HT_{2C} receptor with regard to antipsychotic action is that 5-HT_{2C} antagonism may be functionally opposed to 5-HT_{2A} antagonism.

Meltzer et al. [142] reported that atypical APDs were more likely to be weak 5-HT_{2C} and potent 5-HT_{2A} antagonists compared to typical neuroleptic drugs. There is some evidence for a functional antagonism of these two receptors which may be expressed on the same neurons. Thus, Martin et al. [89] found that ritanserin, a mixed 5-HT_{2A/2C} antagonist, blocked the ability of M100907 to antagonize the effect of MK-801 to increase locomotor activity in mice.

12.10 ROLE OF 5-HT_{1A} RECEPTOR IN ANTIPSYCHOTIC DRUG ACTION: 5-HT_{1A} AND 5-HT_{2A} INTERACTIONS

5-HT_{1A} receptors are located pre- and postsynaptically. The presynaptic 5-HT_{1A} receptor is an autoreceptor located on cell bodies of raphe neurons; stimulation leads to inhibition of firing of 5-HT neurons. Stimulation of postsynaptic 5-HT_{1A} receptors leads to hyperpolarization of pyramidal neurons, opposite to the effect of stimulation of 5-HT_{2A} receptors. Approximately 60% of rat PFC glutamatergic cells were found to have 5-HT_{1A} mRNA, as did approximately 25% of generalized anxiety disorder (GAD)-expressing cells [143]. 5-HT_{1A} receptor agonists have effects similar to 5-HT_{2A} receptor inverse agonists in a variety of functions [144, 145]. A few examples will be given. For example, DOI injected bilaterally into the rat mPFC elicited a dose-dependent head-twitch response. This effect is inhibited by the 5-HT_{1A} agonist 8-OH-DPAT as well as the 5-HT_{2A} inverse agonists M100907 and ketanserin. Ahlenius [146] first suggested that stimulation of 5-HT_{1A} receptors might produce an antipsychotic-like action on the basis of behavioral studies in animals using the direct 5-HT_{1A} agonist 8-OH-DPAT. Subsequent studies demonstrated that 8-OH-DPAT enhanced the antipsychotic-like effect of the D₂/D₃ antagonist raclopride [147] and of haloperidol [148] and antagonized the catalepsy induced by the D₁ agonist SCH23390 in rats [149]. The beneficial effect of 5-HT_{1A} agonists appears to be mediated by inhibition of median raphe serotonergic neurons [150]. Ichikawa and Meltzer [151] demonstrated that 8-OH-DPAT inhibited the ability of clozapine and low-dose risperidone, but not haloperidol, to increase extracellular DA levels in the NAC and the striatum of conscious rats. The effect in the NAC would be expected to enhance the antipsychotic effect of these agents by reducing dopaminergic activity in this region which is believed to participate in the generation of psychotic symptoms. Several atypical APDs are partial agonist at the 5-HT_{1A} receptor, including aripiprazole, bifeprunox, clozapine, ziprasidone, quetiapine, and tiospirone. Their affinities for the 5-HT_{1A} receptor were similar to their affinities at the human D₂ receptor [152]. The ability of atypical APDs to increase DA efflux in the rat PFC and hippocampus is blocked, in part, by WAY-100635, a 5-HT_{1A} antagonist [27, 113, 153, 154]. This effect of 5-HT_{1A} receptors appears to be mediated by 5-HT_{1A} receptors in the PFC as it is blocked by local injection of a 5-HT_{1A} antagonist in the mPFC or by transection of cortical connections to the VTA [155]. These findings suggest that the combination of D₂ antagonism and 5-HT_{1A} agonism should produce an atypical antipsychotic agent [155a]. S16924 is an example of such a compound, and it has atypical properties very similar to those of clozapine in a variety of animal models [137]. The 5-HT_{1A} partial agonist S15535 enhanced the release of ACh in rat mPFC [156]. However, there is little evidence so far that the increase in ACh efflux in mPFC or hippocampus produced by atypical antipsychotic drugs is related to their

direct or indirect 5-HT_{1A} agonism [111, 113]. Bruins et al. [52] have suggested that the balance of activity at D₂ and 5-HT_{1A} receptors is important for the ability of antipsychotic drugs to reverse the deficit in social interaction, a putative model of negative symptoms, in rats.

12.11 ROLE OF 5-HT₆ RECEPTOR

Recent interest in the 5-HT₆ receptor in relation to the action of APDs has been based, in part, on the finding that some APDs, including the atypical APDs clozapine, olanzapine, and sertindole, as well as some antidepressant agents (e.g., clomipramine and amitriptyline), are relatively potent 5-HT₆ receptor antagonists [62, 157–160]. Regional analysis of receptor binding and the expression of 5-HT₆ receptor mRNA revealed the highest levels are found in the striatum, olfactory tubercle, NAC, cerebral cortex, and subfields of the hippocampus [157, 161–164]. Together, these data on expression and localization suggest possible involvement of 5-HT₆ receptors in cognitive, affective, and motor function.

It has been reported that clozapine, the prototypical antipsychotic drug, but not haloperidol, significantly decreased 5-HT₆ receptor expression in all subfields of the hippocampus [165]. As clozapine has a greater effect than haloperidol to improve some aspects of cognition in schizophrenia, it was suggested that downregulation of this receptor in the hippocampus might contribute to the ability of clozapine to enhance cognition [165]. This hypothesis is supported by a series of behavioral studies which demonstrate that the 5-HT₆ receptors may be involved in learning and memory [166–173]. These data suggest that 5-HT₆ receptor stimulation may interfere with learning and long-term memory function and the potential role for 5-HT₆ receptor antagonists for the enhancement of cognition in patients with schizophrenia. The high affinity of some atypical antipsychotic drugs for 5-HT₆ receptors and the localization of 5-HT₆ receptor in limbic and cortical regions of the brain suggest that 5-HT₆ receptors also play a role in the mechanism of action and pathophysiology of some aspects of schizophrenia. Minabe et al. [174] reported that high but not low doses of the selective 5-HT₆ receptor antagonist SB-271046 given acutely suppressed the firing rate of VTA DA neurons. Chronic administration altered the pattern of firing of these neurons, which are highly relevant to schizophrenia in a manner which differed from that of either typical or atypical APDs. It was suggested that clinical study of this type of agent would be of interest to determine its role, if any, in the treatment of schizophrenia. There has been only limited study of the role of the 5-HT₆ receptors in the modulation of DA or ACh release, which might be relevant to their ability to improve cognition, and the results have been inconsistent. 5-HT₆ receptor antagonists have been reported to increase extracellular ACh efflux in the cortex or hippocampus in two studies [175, 176]. However, another 5-HT₆ receptor antagonist RO 04-6790 failed to do so [112].

The 5-HT₆ receptor antagonist SB-271046, which has been shown to be effective in enhancing cognitive function in models of learning and memory [167, 169], had no effect on DA efflux in either the frontal cortex or dorsal hippocampus [177]. However, a recent study reported that SB-271046 produced a significant increase in DA release in the rat mPFC [178]. Pouzet et al. [179] found that SB-271046 dose dependently inhibited *d*-amphetamine-induced suppression of prepulse inhibition

(PPI), consistent with an antipsychotic action. However, Leng et al. [180] found no effect of two other 5-HT₆ antagonists in latent inhibition (LI) or PPI models in which clozapine was active. The 5-HT₆ receptor antagonist *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)-phenyl]-5-chloro-3-methylbenzo-thiophene-2-yl sulfonamide monohydrochloride (SB-258510A) has been reported to produce greater potentiation of amphetamine-induced increase in extracellular DA release in the mPFC than the NAC [181]. Another 5-HT₆ receptor antagonist, SB-271046, potentiated amphetamine-induced DA release in the striatum [182]. Li et al. (in preparation) found that SB-399885, 3 and 10 mg/kg, a selective 5-HT₆ receptor antagonist, had no effect on cortical DA efflux but slightly increased hippocampal DA efflux in freely moving rats. However, SB-399885, 3 mg/kg, significantly potentiated the ability of haloperidol, a D₂ receptor antagonist, at a dose of 0.1 mg/kg, to increase DA release in the hippocampus but not the mPFC. SB-399885 also potentiated risperidone-, 1.0 mg/kg, induced DA efflux in both hippocampus and mPFC. These results suggest that the combined blockade of 5-HT₆ and D₂ receptor may contribute to the potentiation of haloperidol- or risperidone-induced DA efflux in the mPFC and hippocampus. In addition, other microdialysis studies suggest that the 5-HT₆ receptor may interact with DA mechanisms in the rat mPFC as antisense oligonucleotides partially antagonized the fluoxetine-induced cortical DA release [183]. Taken together these data suggest that 5-HT₆ receptors may have modulatory influence on DA efflux in the mPFC and hippocampus and, hence, may contribute to some of the clinical benefits of atypical APDs. 5-HT₆ receptor antagonists may also be useful as augmenting agents to improve cognitive dysfunction in schizophrenia. There is, however, insufficient evidence to conclude that they will be first-line treatments for the psychosis of schizophrenia.

12.12 ROLE OF SEROTONIN RELEASE IN ANTIPSYCHOTIC DRUG ACTION

The antagonism of multiple 5-HT receptors by clozapine would be expected to enhance the release of 5-HT by feedback mechanisms. Ichikawa et al. [184] reported that clozapine (20 mg/kg) and risperidone (1 mg/kg) but not olanzapine significantly increased extracellular 5-HT levels in the NAC and mPFC, respectively, whereas amperozide (1 and 10 mg/kg) increased extracellular 5-HT levels in both regions. Hertel et al. [185] reported similar results with risperidone and suggested that this might be relevant to its ability to improve negative symptoms. The enhancement of 5-HT efflux in the PFC may contribute to the ability of these agents to improve mood disorders and cognition [184]. Bortolozzi et al. [186] reported that clozapine blocked the efflux of cortical 5-HT produced by the hallucinogen DOI in rat brain by both 5-HT_{2A}- and α_1 -adrenoceptor-dependent mechanisms.

12.13 α_2 - AND α_1 -ADRENERGIC MECHANISMS AND ATYPICAL APDS

Many, but not all, of the atypical APDs which are multireceptor antagonists are also potent antagonists of α_1 or α_2 adrenoceptors or both. Thus, risperidone 9-hydroxyrisperidone, clozapine, olanzapine, zotepine, quetiapine, ORG-5222 (asenapine),

sertindole, and ziprasidone are potent α_1 antagonists [60]. Svensson [187] has suggested that blockade of α_1 adrenoceptors by APDs may contribute to suppression of positive symptoms whereas α_2 adrenoceptor blockade may be involved in relief of negative and cognitive symptoms. He postulates that α_1 adrenoceptor blockade may act by suppressing, at the presynaptic level, striatal hyperdopaminergia, and α_2 adrenoceptor blockade may act by augmenting and improving prefrontal dopaminergic functioning. Prazosin, an α_1 adrenoceptor antagonist, can, in the presence of weak D_2 receptor blockade, block the conditioned avoidance response [188] and has, similar to clozapine and other atypical APDs, been shown to increase DA efflux in the shell but not the core of the NAC, signifying a limbic rather than a striatal effect of α_1 antagonism [189]. These authors also suggested that α_1 antagonism may explain the atypical properties of sertindole which has as high an occupancy of D_2 receptors as typical APDs [190]. All of the atypical agents mentioned above are also potent α_2 antagonists with the exception of zotepine and sertindole. Kalkman et al. [191] have raised the possibility that the α_{2C} subtype may be particularly relevant to the anticataleptic actions of clozapine and iloperidone. However, McAllister and Rey [192] were unable to reverse the effects of loxapine or haloperidol on catalepsy with α_2 antagonists and showed that the effect of clozapine to reverse loxapine-induced increase in catalepsy was due to its anticholinergic rather than its adrenoceptor blocking properties.

The addition of idazoxan, an α_2 antagonist, to fluphenazine, a typical neuroleptic, was reported by Litman et al. [193] to have efficacy comparable to clozapine in a small group of neuroleptic-resistant patients with SCH. These results need to be replicated. Polymorphisms of the α_1 and α_2 receptors have been reported not to predict response to clozapine [194]. In this regard, it is of interest that idazoxan has been shown to preferentially increase DA efflux in the rat mPFC by an action at the terminal area [195]. This effect appears to be independent of dopaminergic activity. Subsequently, it was reported that the combination of raclopride, a D_2 antagonist, and idazoxan [196] produced a dose-dependent increase in NE in the mPFC of rats, as does systemic administration of clozapine, risperidone, ziprasidone, and olanzapine but also haloperidol [197]. This effect was closely coupled to the increase in DA efflux. Stimulation of α_2 receptors in cortex has been postulated to improve some types of cognitive function, for example, executive functions [198, 199]. Atypical antipsychotic drugs block α_2 receptors as noted above and would be expected to compromise cognitive function on this basis. However, these drugs also enhance the release of NE along with DA, which could overcome the effects of α_2 blockade. In fact, the atypical antipsychotic drugs have their least beneficial effect on executive function [9].

12.14 GLUTAMATERGIC MECHANISMS

Glutamate is the predominant excitatory neurotransmitter in the mammalian CNS. There are two types of glutamate receptors (GluRs): ionotropic GluRs, which are postsynaptic, multimeric ligand-gated ion channels classified into three groups related to group-selective agonists—NMDA, amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainite receptors—and metabotropic GluRs (mGluRs), which are members of family C of the G-protein-coupled receptors. None of the

currently available APDs have a high affinity for any of the ionotropic or metabotropic GluRs. Because of some similarities between the behavioral effects of NMDA receptor antagonists PCP and ketamine to the cognitive, negative, and positive symptoms of schizophrenia, it has been suggested that hypoglutamatergic function might contribute to schizophrenia [15, 200]. Laruelle et al. [201] summarized data for dopaminergic–glutamatergic interactions and proposed that hypoglutamatergic cortical function might lead to increased mesolimbic dopaminergic stimulation while diminution of mesolimbic dopamine function might reverse hypoglutamatergia. There have been numerous *in vivo* microdialysis studies of the effect of haloperidol and clozapine, as well as other antipsychotic drugs, to modulate the efflux of glutamate in cortex or striatum. The results are very mixed, reflecting differences in dosages, duration of treatment, use of anesthesia, and difficulty in separating glutamate efflux that is neuronally based from that due to the amino acid compartment [202]. Chronic treatment with haloperidol for six weeks followed by its withdrawal for four days elevated the basal, but not the veratridine-stimulated, extracellular levels of glutamate and aspartate [203] and enhanced the activity of cortical neurons. In contrast, six-week treatment with clozapine lowered both the basal and the stimulated levels of glutamate and aspartate but had no effect on the activity of cortical neurons. In a study of the effect of these two antipsychotic drugs on glutamate release from nerve terminals isolated from rat PFC, Yang and Wang [204] found that both haloperidol and clozapine inhibited glutamate release by ion-channel activities which influence nerve terminal excitability. At the present time, this effect of antipsychotic drugs on glutamate release cannot be related to their actions with any degree of confidence.

However, behavioral studies involving two models of cognitive impairment in schizophrenia, PPI and LI, provide more robust evidence that glutamatergic mechanisms may be important to the action of antipsychotic drugs. PPI of the startle response provides a measure of the ability of the organism to attenuate its response to repeated sensory input. The diminished response to sound- or air pulse-induced startle is usually used to test this reflex. Latent inhibition is a measure of the capacity to ignore irrelevant (nonreinforced) stimuli. Both PPI and LI are disrupted in schizophrenia. Treatment with NMDA receptor antagonists such as MK-801 or PCP can disrupt PPI and LI. Atypical antipsychotic drugs such as clozapine are more effective than typical antipsychotic drugs to reverse PPI [205, 206] or LI [207]. A recent study found no difference between typical and atypical APDs in reducing startle magnitude and increasing PPI in a mouse line which expresses low levels of the NMDA R1 subunit of the NMDA receptor [208]. The ability of drugs to reverse PCP-induced PPI disruption is correlated to their affinity for 5-HT_{2A}, not D₂ receptors [209]. Interestingly, the 5-HT_{2A} antagonist M100907 and the α_1 -adrenergic antagonist prazosin are also effective to reverse PPI deficits induced by NMDA receptor antagonists [205] but the α_2 antagonist RX821002, the M₁/M₄ muscarinic antagonist pirenzepine, or the GABA-A antagonist picrotoxin had no effect on basal or PCP-impaired PPI in mice [210]. As previously mentioned, 5-HT_{2A} receptors on cortical glutamatergic pyramidal neurons and GABAergic interneurons may have a key role in modulating glutamatergic function [143]. It has been suggested that the hallucinogenic action of 5-HT_{2A} agonists such as DOI is mediated by mGluR2/3-induced glutamate release as the head shakes induced by DOI are blocked by the mGluR2/3 agonist LY354740 and potentiated by the mGluR2/3 antagonist

LY341495 [211]. The combination of raclopride, a D_2 antagonist, and idazoxan, an α_2 antagonist, also was ineffective to block PCP-induced disruption of PPI [212]. Neonatal ventral hippocampal (NVH) lesions in rats induce behavioral abnormalities in adult rats that have some correspondence to cognitive deficits and negative symptoms in patients with schizophrenia, including deficits in PPI. Clozapine, olanzapine, and risperidone, but not haloperidol, reversed the deficit in PPI produced by an NVH lesion, possibly by enhancing glutamatergic function [213].

There is some evidence for a possible role of mGluR5 in schizophrenia [214]. mGluR2 agonists [215, 216] and positive allosteric modulators of the mGluR5 [217] have been shown to have activity in various animal models of antipsychotic activity, but there is no direct evidence that the current generation of atypical antipsychotic drugs act through either mechanism. Thus, neither clozapine nor raclopride were able to attenuate the PPI deficit in mGluR5 knockout mice [214].

12.15 CHOLINERGIC MECHANISMS

The concept of muscarinic agonism as a first-line treatment for schizophrenia was initiated by studies with xanomeline, which is a M_1/M_4 agonist or partial agonist. It was found to have some antipsychotic effects in patients with Alzheimer's disease or schizophrenia [218]. Preclinical studies with xanomeline and a closely related compound sabcomeline indicated it had the profile of an atypical APD, blocking conditioned avoidance response and producing minimal EPSs [219, 220]. Sabcomeline and possibly xanomeline also have indirect effects on D_2 receptor binding in the mouse striatum, as revealed by changes in the availability of D_2 receptors [221]. NDMC, the major metabolite of clozapine, has been reported to be an effective M_1 agonist in vitro [median effective concentration (EC_{50}) of 100 nM with 80% efficacy (relative to carbachol)] as well as in vivo [222, 223]. The M_1 agonist activity of NDMC is blocked by anticholinergic drugs and clozapine [222, 223]. These results confirm that NDMC is a potent, efficacious, M_1 receptor agonist, distinguishing it from the M_1 receptor antagonist properties of clozapine. No other antipsychotic has potent M_1 agonist properties [223, 224]. Clozapine is, in fact, a very weak partial agonist at M_1 receptors, a more efficacious agonist at M_2 and M_4 receptors, and to lack agonist activity at M_3 and M_5 receptors. NDMC also displayed high-potency interactions with all five human muscarinic receptors, but with increased agonist efficacy at M_1 , M_4 , and M_5 receptors when compared to clozapine. In contrast, olanzapine and *N*-desmethyloanzapine, both structurally related to clozapine and NDMC, lack agonist activity at human muscarinic receptors [223].

Li et al. [236] have reported that NDMC enhances DA and ACh efflux in the mPFC and hippocampus. Clozapine blocks the effects of NDMC on cortical and hippocampal ACh efflux and on hippocampal DA efflux [82]. The M_1 antagonist telenzepine also blocked the effect of NDMC on DA and ACh efflux. These results suggest that clozapine, acting through its predominant metabolite NDMC, may function as a direct-acting muscarinic receptor agonist in vivo. During pharmacotherapy with clozapine, the agonist actions of NDMC would be attenuated by the antagonistic actions of the parent compound. Thus, high NDMC levels, and particularly high NDMC/clozapine ratios, would increase agonist efficacy at muscarinic receptors, as predicted by mass action and by agonist/antagonist mixing

studies [225]. Clinical data support this notion. Not only does clozapine therapy usually lack the traditional anti cholinergic side effects of dry mouth, blurred vision, and urinary retention common to classical muscarinic antagonists, but also it is unique in its ability to frequently produce sialorrhea, an effect that can be blocked by the muscarinic antagonist pirenzepine [226]. Thus, the muscarinic receptor agonist activity of NDMC likely mediates this peripheral effect. The muscarinic agonist properties of NDMC may underlie some of the unique central effects of treatment with clozapine. If this hypothesis is correct, NDMC/clozapine ratios should be a better predictor of therapeutic response to clozapine, particularly for cognition, than absolute clozapine levels. As predicted, higher NDMC/clozapine ratios predicted improvement in multiple measures of cognition as well as negative symptoms [223]. Some other recent reports [227, 228] all suggest that the NDMC/clozapine ratio is a better predictor of clinical response to clozapine than clozapine levels alone and support the hypothesis that NDMC is a critical mediator of clozapine action.

12.16 NEUROKININ 3 RECEPTORS

The possible involvement of neurokinin 3 (NK₃) receptors in antipsychotic drug action has been recently reviewed [229]. The NK₃ antagonists osanetant and talnetant have been tested in schizophrenia. Osanetant showed some efficacy to treat positive symptoms with no evidence of EPSs in randomized, placebo- and haloperidol-controlled trials, indicating it met the criteria for an atypical APD [78]. Talnetant is in phase 2 testing but no results have been reported as of yet. Should either or both of these two agents prove clinically effective as monotherapy or as add-on therapy, it would be a major advance in the treatment of schizophrenia. Preclinical data suggest that NK₃ receptor antagonism achieves its antipsychotic efficacy by inhibition of dopaminergic activity via an indirect mechanism [230].

12.17 NEUROGENESIS

The evidence for the loss of neurons and decreased neuropil in schizophrenia [231] provided the initial impetus for the investigation of a possible role of antipsychotic drugs in schizophrenia [232]. In a review of the effects of antipsychotic drugs on neuroplasticity, these authors concluded that the ability of long-term treatment with antipsychotic drugs to induce anatomical and molecular changes in the brain may be relevant to their antipsychotic properties. Imaging studies which indicate that atypical but not typical antipsychotic drugs slow the loss of cortical volume in schizophrenia is compatible with the view that neurogenesis may be, at least, a component of the action of atypical antipsychotic drugs [233]. Preclinical studies have added some evidence for this hypothesis. Atypical but not typical APDs risperidone and olanzapine increase the number of newly generated cells in the subventricular zone [234]. Wang et al. [235] reported that three-week treatment with olanzapine but not haloperidol produced slight evidence for cortical and dorsal striatal neurogenesis but none in the NAC. They concluded that neurogenesis did not play a significant role in the effects of olanzapine on cortical function. Further study in this regard is still of interest, however.

12.18 CONCLUSIONS

Atypical APDs are those APDs which produce an antipsychotic effect at doses which cause minimal EPSs compared to typical APDs. Any other clinical feature of the atypical APDs is not part of the core definition, even if the bases for the additional clinical effect are related to the same pharmacological principles. Thus, lack of prolactin elevation is not a core feature of an atypical APD, even though it, like low EPSs, may be related to potent 5-HT_{2A} antagonism relative to weaker D₂ antagonism. The advantage to patients of having antipsychotic drugs with reduced risk parkinsonism and tardive dyskinesia are adequate reasons for making these drugs the first-line treatment for schizophrenia and bipolar disorder. There are now two established classes of atypical APDs: multireceptor antagonists which have a common serotonergic profile (e.g., clozapine) and partial DA agonists (e.g., aripiprazole). All clinically viable examples of these two classes of agents involve both a dopaminergic and serotonergic profile. 5-HT_{1A} partial agonism may be a functional substitute for 5-HT_{2A} antagonism. There is strong evidence for the role of 5-HT_{2A} receptor antagonism and 5-HT_{1A} partial agonism as a sufficient if not necessary feature of an atypical APD, in conjunction with no more than moderate D₂ receptor blockade whether it be the result of direct blockade or partial agonism. Studies are underway to determine if selective 5-HT_{2A/2C} antagonists are sufficient to treat some forms of psychosis without supplemental D₂ receptor blockade. 5-HT₆ antagonism may also facilitate some of the clinical advantages of those atypical APDs, which are potent 5-HT₆ antagonists. 5-HT_{2C} receptor blockade is a feature of some atypical APDs, but its contribution to their mechanism of action is unclear at the present time. Rapid dissociation from the D₂ receptor is not a core feature of most examples of these two classes of APDs but may contribute to the low EPS profile of clozapine and quetiapine. There are five other groups of drugs which may be atypical APDs. These include substituted benzamides, of which there are two main examples, amisulpride and remoxipride; selective 5-HT_{2A/2C} inverse agonists; D₂/5-HT_{1A} partial agonists; NK₃ antagonists; and M₁ agonists or partial agonists, of which there is only one available and that is not specific for M₁. These mechanisms of atypicality are promising and need to be further studied. There are no atypical APDs, as of yet, which have come from intensive study of D₁, D₃, or D₄ mechanisms. There are also no atypical APDs which primarily act through glutamatergic mechanisms, despite intensive effort to date to develop them and the fact that the multireceptor antagonists have been shown to act, in part, by modulating glutamatergic pathways.

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13

INTRODUCTION TO ADDICTIVE DISORDERS: IMPLICATIONS FOR PHARMACOTHERAPIES

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13.1	History of Pharmacotherapies	451
13.2	Vulnerability to Develop a Specific Addiction	457
13.3	Reward, Modulation, and CounterModulation of Reward and their Role in Specific Addictions	458
	Acknowledgment	459
	References	459

13.1 HISTORY OF PHARMACOTHERAPIES

Although a few clinical and also laboratory-based research groups had addressed specific problems related to addictions from 1890 to 1964 and over that same time period a variety of different approaches, including some use of diverse chemicals as “medications,” by the early 1960s there was still no clear understanding of the bases of addictions and no consistent approach to their management, other than incarceration. Our original research team first coalesced at The Rockefeller University in very early 1964, led by Vincent P. Dole, a clinical and laboratory investigator of metabolism who up to that time had focused on hypertension and lipid disorders. Included in the group was the late Marie Nyswander, a psychiatrist who had written the very sensitive book *The Addict as a Patient*, and also this author (then a second-year postgraduate trainee having completed medical school and aimed toward a life in academic research medicine). The first task on which we focused was a definition and hypothesis related to addiction. After studying all available written materials of a variety of types and, probably more importantly, getting directly involved in interviewing scores of primarily heroin addicts, a paradigm shift in thinking very rapidly evolved. Thus, by the time we became immersed in our basic clinical research studies in the early spring of 1964 at the Rockefeller Hospital [primarily a National

Institutes of Health (NIH)–supported General Clinical Research Center], we formulated the following hypothesis: “Heroin (opiate) addiction is a disease—a ‘metabolic disease’ of the brain, with resultant behaviors of ‘drug hunger’ and drug self-administration, despite negative consequences to self and others. Heroin addiction is not simply a criminal behavior or due alone to any social personality or some other personality disorders” (paraphrased from the original research paper of 1966 covering clinical laboratory-based research performed in 1964 and presented before the Association of American Physicians (AAP) in 1966 [1, 2]. At that time, Dole decided to change his focus to the enormous social problem of heroin addiction, which he clearly saw as a medical problem with definable “metabolic” bases.

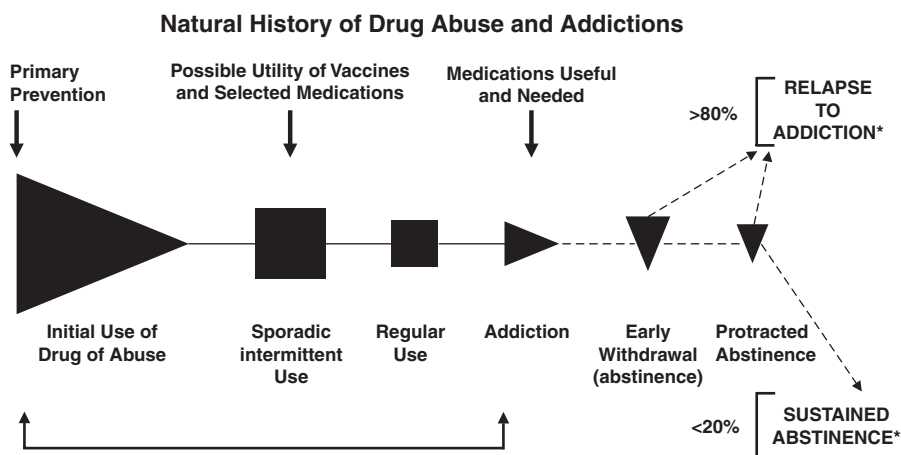
In the first few months of our research together, we had two simple goals. The first was to develop a pharmacotherapy to treat heroin addiction combined with the best behavioral therapeutic approaches, including rigorous but humane counseling as well as psychological and psychiatric and medical care as needed. The second goal was to attempt to define the physiological bases of such treatment if in fact we found one which was successful. At the time of our earliest work, there were no analytical laboratory techniques to measure plasma or even urine levels of methadone, heroin or its major metabolite morphine, or any other opiate like compound, and there were no precise molecular definitions for sites of action of these opiates. Nevertheless, it was known by qualitative work that heroin was metabolized primarily to morphine, at that time (and still) the most commonly used opioid for analgesia purposes which was administered only by parenteral (intravenous or subcutaneous) routes because of poor bioavailability after oral administration. The majority of heroin addicts used heroin by the intravenous route, although a few began use by a nasal route (“snorting”) and later by a subcutaneous route (“skin popping”) and ultimately by the intravenous route (“main lining”). These three routes of administration are still the most commonly used for many drugs of abuse, with an additional route of smoking or snorting (“freebase”). Extensive laboratory and also clinical work had been conducted on opioid analgesics. The concept of specific opiate receptor binding sites had been introduced by three groups in particular: Martin at the U.S. Public Health Service (PHS) Hospital, then in Lexington, Kentucky; Collier, in England; and our group, headed by Dole, at The Rockefeller University. All three research teams postulated that there were specific opiate receptors. The original work done to pursue that concept, from the 1960s to 1973, has been extensively reviewed, including, in part, in some of our own reviews [3–10].

In our first search for a pharmacotherapeutic agent, we wanted a medication which would be orally effective, to simplify pharmacotherapeutics; a compound with a slow onset of action, since even at that time rapid onset of action was directly related to the euphoria-producing effects of a drug of abuse (and now appreciated to be directly related to the “reinforcing” or “rewarding” effects of a drug of abuse); and a compound with a slow offset of action, to prevent the rapid precipitation of the physiological signs and symptoms of withdrawal in a tolerant and physically dependent individual, since opiates and some other addictive agents, such as barbiturates and alcohol, do produce physical as well as psychic dependence. Early on in our work, we also developed the concepts that a pharmacological agent should achieve two important therapeutic goals: first, a medication should reduce or eliminate all signs and symptoms of opiate withdrawal and, second, it should significantly reduce or eliminate all significant illicit drug “craving” and seeking and also self-administration.

There were then a very limited number of opiate analgesics approved for administration by the oral route. One of these was an infrequently used analgesic, methadone, a very simple synthetic organic diphenyl-heptanone, which had been synthesized by German chemists during World War II but which never had been studied in the clinic. When it was brought to the United States, two pain groups studied this medication and found that when delivered to opiate-naïve individuals, its duration of action with respect to analgesia was approximately the same as morphine, but further, when multiple doses of methadone were given per day, as was the practice with the short-acting available morphine, objective signs of respiratory depression began to appear, suggesting an impending opiate overdose. However, all of these studies were conducted in opioid-naïve subjects; many years later, it became fully appreciated that methadone is the opioid analgesic of choice when delivered orally in most cases of chronic pain, such as cancer-related pain, due to its long duration of action and minimal peak effects and also since it is possible to add additional short-acting opiates to sustained methadone treatment to manage recrudescence of acute pain. However, such use is done in persons receiving opiates on a chronic basis; methadone is not usually the appropriate first opiate to use in analgesia management in opiate-naïve individuals.

In a series of studies which have been published and reviewed extensively elsewhere, we found that methadone is orally effective, that beginning at low doses not to exceed 20–40 mg/day in long-term opioid-dependent heroin addicts, and then gradually increasing the doses over a period of four to eight weeks to a full treatment dose of 80–120 mg/day, no euphoric effects were noted and signs and symptoms were completely prevented [1, 2]. Further, in rigorous four-week studies, each conducted in a double-blind random-order Latin square design, we determined that a dose of 80–100 mg/day of methadone delivered orally, as just described, completely prevents all perception as well as objectively measured signs and symptoms of a superimposed short-acting opiate such as heroin or hydromorphone. In further single-blind studies, we found that to overcome this “narcotic blockade,” it is essential to superimpose 200 mg of pure heroin intravenously on the daily oral dose (80–120 mg) of methadone treatment [1–10]. During the next eight years, two different groups developed sensitive analytical techniques using gas liquid chromatography to measure plasma as well as urine levels of methadone and its metabolites. Both our laboratory and that of Charles Inturrisi published independent studies showing that the half-life in humans of racemic methadone is 24 h. (of note and importance for laboratory-based investigators, we learned in other studies that the half-life of methadone in rats is around 90 min and in mice around 60 min) [11–14].

In 1964, after the initial clinical laboratory studies, prospective studies were initiated and later special studies were carried out to determine the physiological effects, medical safety, and effectiveness of methadone; other studies were published in the early 1970s and led to the Food and Drug Administration (FDA) approval of methadone for use in the maintenance treatment of heroin and other short-acting opiate addiction by 1973 [15–19]. Later, long-term follow-up studies were conducted of a randomly selected group of methadone-maintained patients from the early treatment cohorts previously studied who had been in continuous treatment for 11–18 years [20]. Thus, the medical safety as well as physiological effects of methadone were rigorously studied from its first use, with the first subject included in data on both safety and effectiveness (reviewed in [1–10; 13–28]).



SHORT ACTING OPIOIDS: 1 in 3 to 1 in 5 who ever self-administer progress to addiction

COCAINE: 1 in 8 to 1 in 15 who ever self-administer progress to addiction

ALCOHOL: 1 in 8 to 1 in 15 who ever self-administer progress to addiction

* with no medications

Figure 13.1 Natural history of drug abuse and addictions.(Adapted from Kreek et al., *Nature Reviews Drug Discovery*, 2002.)

The regulations covering methadone maintenance treatment which were promulgated in 1973 have been modified only once, in 1983 [26]. For entry into methadone maintenance treatment, these regulations define far more severe heroin addiction than the criteria in the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) of “opiate dependence” or addiction. The federal regulation guidelines still demand that a person be regularly using heroin or another short-acting opiate daily for one year or more prior to entry in methadone maintenance treatment. In numerous laboratory models and in some careful clinical studies, it has been shown that each major drug of abuse administered on a chronic basis causes specific objective changes in the brain and brain function. Also, the relapse rate after medication-free treatment remains extraordinarily high (over 80%) in persons who have been addicted for that length of time. Therefore the Institute of Medicine of the National Academy of Sciences, after a series of extensive meetings, recommended in 1995 that the guidelines be altered to demand only six months or even less of daily self-administration prior to entry into methadone, or λ - α -acetyl methadol (LAAM), maintenance treatment [26]. This regulation, however, has still not changed, but most early federal regulations have been dropped. The previous regulation of the initial maximum daily oral dose of methadone was 120 mg/day for many years, subsequently increased to 150 mg/day and currently with no defined upper limit because of the dramatic increase in the purity of heroin. Also, office-based maintenance treatment using methadone is allowed at the federal level now; the only provision is that initially addicts have to be admitted by a standard opioid treatment clinic, that is, a methadone maintenance or other clinic configured by the original methadone guidelines prior to transferral to a doctor’s office for continuing care. However, the duration that subjects have to be in such an organized clinic and the

achievements made in their steps to recovery have not been defined. We have reviewed these recent guidelines elsewhere [15]. These regulations are still far more restrictive than those which currently pertain for another opioid partial agonist treatment, that is, treatment with the partial agonist buprenorphine, with or without the combination of naloxone.

Overall, at this time, there are approximately 212,000 persons in methadone maintenance treatment in the United States and over half a million worldwide. The voluntary retention in good clinics which use adequate doses of methadone (usually 80–150 mg/day), along with adequate counseling and psychological and psychiatric care remains over 50%, with excellent clinics still achieving over 80% voluntary retention at one year [3, 4, 9, 10, 29]. The actions of methadone treatment were early established to include prevention of withdrawal symptoms and reduction or elimination of “drug hunger” or drug craving. It was established in early clinical laboratory studies and reproduced by many groups over the last 40 years that methadone blocks the euphoric effect of superimposed short-acting narcotics. Also, further studies, including our prospective and special medical studies and medical safety and many subsequent studies, have shown that steady-dose methadone treatment allows normalization of physiology disrupted by long-term heroin use. The mechanism of action was hypothesized and then established by the time the specific opiate receptors were clearly defined to exist in 1973. It was established by then newly developed analytical techniques that methadone provides steady levels of this opioid at the specific opiate receptor sites. Subsequent work in the 1970s established that there are μ -opioid receptors where heroin, morphine, and methadone primarily act. More recent work by several groups have shown that methadone is a full μ -opioid agonist, not a partial agonist, and further that after binding to μ -opioid receptors methadone undergoes immediate internalization bound to the receptor, similar to the physiological handling of β -endorphin, enkephalin, and other μ -opioid receptor-directed

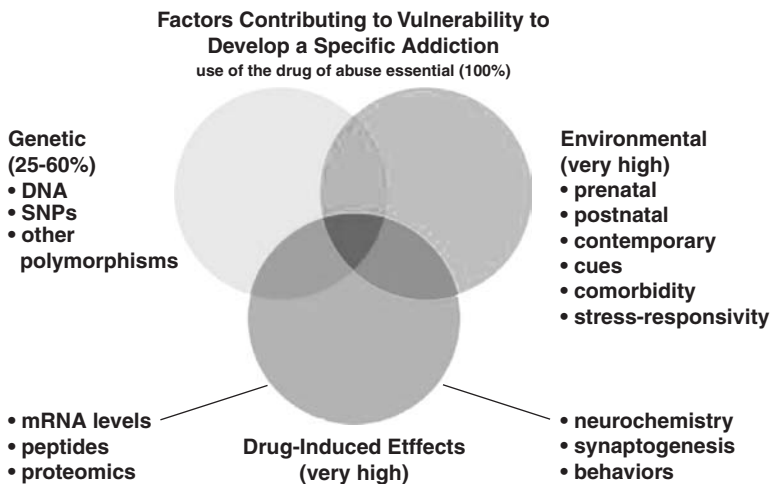


Figure 13.2 Factors contributing to vulnerability to develop a specific addiction. (Adapted from Kreek et al., *Nature Reviews Drug Discovery*, 2002.) (See color insert.)

endogenous opioids. This is in sharp contrast with most exogenous synthetic or natural plant opioids, which bind to the receptors but then prevent the receptor from rapidly internalizing. In addition, it has been shown both in laboratory studies and more recently in human studies that the two enantiomers of methadone have modest *N*-methyl-D-aspartate (NMDA) receptor antagonism which would be expected to slow or attenuate the development for opioid tolerance. Our early stable isotope mass spectrometry research showed that the two enantiomers of methadone are metabolized at very different rates, with the active enantiomer l(R) having a half-life of over 36 h in humans and the inactive d(S) enantiomer having a half-life of around 16 h, findings confirmed more recently by several groups. In contrast, it has been shown that heroin has a half-life of only 3 min in humans. Its first and active metabolite, 6-acetyl-morphine, has a half-life of 30 min in humans, and its major metabolite, morphine, and its major active metabolite, morphine-6-glucuronide, have half-lives of 4 and 6 h, respectively (reviewed in [4, 9, 10, 13, 15, 16]).

Several other pharmacological treatments for opiate addiction have been developed, including LAAM, a full agonist which is biotransformed to two active metabolites conferring an even longer half-life in humans than methadone, and buprenorphine, a μ -opioid receptor partial agonist which must be administered sublingually because of its essentially complete “first-pass” effect, that is, biotransformation by the liver after oral administration, and also an opioid which gives a rapid onset of action if used or abused by a parenteral route. This led to the development of a better sublingual formulation, combining buprenorphine with naloxone, which has no adverse effect when administered by this route but would blunt the peak effects of buprenorphine if this formulation were abused in a parenteral route. Each of these compounds has been studied extensively and is effective in the long-term, maintained management of heroin and other slow-acting opiate addiction. However, the maximum dose of buprenorphine which gives any increased opiate effect in humans, because it is a partial agonist, is around 24–32 mg sublingually, equivalent to a maximal 60–70 mg of methadone, a lower dose than shown in many studies over 40 years to be essential for most unselected heroin addicts. The use of each of these compounds in treatment of heroin and other opiate addiction has been reviewed in many articles and will be addressed in Chapter 19 (reviewed in [8, 10, 13, 15, 16, 30]).

Figure. 13.1 presents a history of the development of drug abuse and drug addiction after first self-exposure. It has been well established that primary prevention can be extremely effective, but it is most effective prior to any initial use of a drug of abuse. In the future, vaccines of a variety of types (several now under development specifically for nicotine, cocaine, and heroin) and also some selected medications may have possible utility after the initial sporadic, intermittent use of a drug of abuse occurs. However, regular use, although continuing in only a limited proportion of those who initiate use of a drug of abuse, leads in most cases to addiction. At that point, medications are useful and, in fact, needed. If no targeted specific medications are available, repeated studies over the past 70 years (such as the first outcome studies performed at the PHS facility for opiate addicts in Lexington, Kentucky, after its opening in the mid-1930s) have shown that over 80% of persons meeting any established national (e.g., DSM-IV) or international criteria for addiction will ultimately relapse to an addiction and, in most cases, to the specific initial predominant addiction. Again, according to repeated studies in which the proper denominator is

used, that is, all persons entering into any form of management or treatment, less than 20% will respond with sustained abstinence to drug-free, medication-free treatment for one year or more. Therefore, targeted specific medications are needed, and, further, many studies have shown such medications are most effective, by far, when administered in conjunction with behavioral treatments, including counseling and psychological or psychiatric care, as needed (e.g., [4, 5, 8–10, 15, 16, 26, 28–35]). Reviews of many different epidemiological studies over the years as well as our own meta-analyses have shown that approximately 1 in 3 to 1 in 5 who ever self-administer a short-acting opiate, such as heroin, will progress to addiction; approximately 1 in 8 to 1 in 15 who ever self-administer cocaine will progress to cocaine addiction, and roughly 1 in 8 to 1 in 15 who ever self-administer alcohol will progress to alcoholism (see Fig. 13.1 [in 16]).

The chapters in this section will address specific drugs or classes of drugs. In each case, what is known about the nature of each addiction at a molecular, neurobiological, behavioral, and/or global level will be discussed followed by proven or projected interventions with a focus on pharmacotherapeutic interventions either available now or under various stages of development. The chapters will focus on a specific type of addiction, including alcohol abuse and addiction, nicotine abuse and addiction, psychostimulant abuse and addiction, 3, 4 methylenedioxymethamphetamine (MDMA [“Ecstasy”]) and other “club” drug abuse and addiction, and marijuana abuse and addiction.

13.2 VULNERABILITY TO DEVELOP A SPECIFIC ADDICTION

We have defined three domains of factors which may contribute to developing a specific addiction (see Fig. 13.2). These include personal factors such as comorbidity with psychiatric or medical illnesses and also responsivity of an individual along with prenatal and postnatal events, contemporary events, cues or set and setting, and environmental factors. The second domain comprises drug-induced factors; we hypothesized in the mid-1980s that each drug of abuse would profoundly alter molecular events in the brain. Our laboratory and many others worldwide now have joined in an effort which has clearly defined that each major drug of abuse, when administered on a chronic (though usually not acute) basis and in an addiction-like pattern or mode, will cause significant and persistent changes in the brain, including messenger RNA (mRNA) levels, resulting peptides, proteomics, integrated neurochemistry, synaptogenesis, possibly even neurogenesis, and resultant behaviors. Further, our group and others have shown that although many acute effects occur, many of these rapidly disappear and new alterations occur after subacute or chronic administration, some of which may be long-term persistent (or possibly permanent) after cessation of exposure to a drug of abuse. Third and finally, it has been clearly shown that genetic factors play a role and make a 25–60% contribution to the risk for development of specific addictions, as well as any addiction. These genetic factors undoubtedly will include a variety of single nucleotide polymorphisms (SNPs) and other types of polymorphisms of many genes acting in concert to increase vulnerability to develop addiction [36–46]. Essentially all investigators in the area hypothesize that addictions will be complex, not just simple, genetic disorders, that is, involving multiple variants of multiple genes. Moreover, it should be clearly understood that different individuals will have different combinations of these gene

variants, in part due to the profound allelic frequency differences of many variants in different ethnic groups, in part due to genetic factors contributing to comorbidity, including differences in drug metabolism as well as psychiatric and some types of medical comorbidity, and in part, because many of the variants will ultimately be shown to be functional, may have very different kinds of impacts on the resultant peptides. Gene variants could include actually changing peptide structure if the gene variant is in the coding region of a gene, or altering amounts of message, and therefore peptide amounts produced, especially if the variants are in critical locations of the 5' promoter region as well as certain intronic and 3' regions. Thus, environmental, drug-induced, and genetic factors all may contribute to the vulnerability to develop a specific addiction in an individual (see Fig. 13.2). These changes have been discussed in detail in many recent and earlier review articles ([3, 4, 6–10, 14, 16, 24, 25, 27, 28, 31–34, 36–59]).

13.3 REWARD, MODULATION, AND COUNTERMODULATION OF REWARD AND THEIR ROLE IN SPECIFIC ADDICTIONS

It has been well established by innumerable groups that drugs of abuse have rewarding or reinforcing effects in the context of use which strengthen the perception and, more importantly, the memory of euphoria or pleasure. In addition, each of these effects uses some natural molecular–neurobiological neurotransmitter or transporter systems, including established neural pathways. However, distortions develop in both the absolute and relative amounts of each neurotransmitter, receptor, and downstream single-transduction event component as well as the neuropathways, and possibly synaptogenesis and neurogenesis get altered in the development of “reward,” with the associated processes of learning and memory. The natural modulators of each of these systems begin to play a role and they too may become exaggerated, leading to countermodulation of the so-called “rewarding” effects of each drug of abuse, which also contributes to the abnormal status which often persists in the drug-free and medication-free state.

Using a variety of models, much work has been done worldwide on what may be the specific components of both reward, and modulation and countermodulation of reward. Clearly, the majority of studies have pointed to the intermediate-acting monoamine neurotransmitter dopamine as being central to reward. However, two other monoaminergic systems, the serotonin and norepinephrine systems, have been shown to be also involved. Our laboratory and many others have also shown unequivocally that the μ -opioid receptor system is involved in reward. There are increasing numbers of studies showing that whereas for some drugs of abuse the dopaminergic system may dominate, for other drugs of abuse the μ -opioidergic system may predominate. Further, it has been shown that the dopaminergic system is involved in some of the effects of all drugs of abuse, but, unexpectedly, our group and others have shown that the endogenous μ -opioidergic system is also involved to a certain extent in most, if not all, of the rewarding properties of drugs of abuse (reviewed in [6–9, 14, 16, 24, 27, 28, 32–34, 36, 38–41, 44]).

Immediate modulators include the usual rapid-acting and stimulatory neurotransmitter systems, including, but not exclusively, the γ -aminobutyric acid (GABA)–ergic and glutamatergic systems, as well as the much slower neuropeptide

cannabinoid systems and others. However, distortion of these systems also occurs. Our group and others have shown that the natural dynorphin opioid peptides acting at the κ -opioid receptors play a major role in countermodulation through their ability to reduce dopaminergic tone. Similarly, orphanin-nociceptin FQ, acting at its specific opioid-like but non-opioid receptors, plays a role similar to that of dynorphin acting at the κ -opioid receptors, with modulation of dopaminergic tone. Further, this latter system has also been shown to alter stress responsivity (see below).

One of the initial hypotheses of our laboratory was that an atypical response to stress and stressors may contribute to the persistence of and relapse to a specific addiction, and further, in some individuals, atypical stress responsivity existing a priori on a genetic or environmental basis may contribute to the initial acquisition of an addiction [6, 9, 10, 14, 18, 19, 21, 24, 25, 28, 47–51]. Many studies from our laboratory in humans, including genetics studies as well as basic molecular neuroendocrine studies, have given strong support to this hypothesis. Similarly, in work from our laboratory and innumerable other laboratories, using a variety of animal models, it has been shown that stress responsivity plays a central role both in the acquisition of drug abuse and in the relapse to self-administration or “reinstatement” of drug abuse. Again, these studies have been reported in both early and very recent papers from our laboratory and in some of our review articles [47–59].

Therefore the rewarding or reinforcing effects of drugs of abuse coupled with modulation and countermodulation in the context of specific environments and the specific genetic fabric of each individual and especially with the predictable chronic drug effects which may persist after drug use has ceased all contribute to the acquisition, persistence and perpetuation of an addiction, and some contribute to learning and memory and thus behaviors in the medication-free, drug-free state, despite aggressive counseling and behavioral treatments. These persistent changes in the brain and resultant behaviors may lead to relapse and thus perpetuation of addiction; these topics will be considered in the chapters that follow. Each site of action and alteration provides potential targets for pharmacological intervention as well as increasingly selective behavioral interventions. However, it is now clear that specific pharmacotherapies will be needed for most persons suffering from specific addictive diseases.

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14

DOPAMINERGIC AND GABAERGIC REGULATION OF ALCOHOL- MOTIVATED BEHAVIORS: NOVEL NEUROANATOMICAL SUBSTRATES

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14.1	Introduction	466
14.2	Optimal Animal Model to Study Neuroanatomical Substrates of Alcohol Self-Administration Behaviors	466
14.3	Integrating P Rat Line with Appropriate Behavioral Paradigms and Neuroanatomical Studies to Make Inferences About Novel Neuroanatomical Substrates of Alcohol Self-Administration Behaviors	467
14.3.1	Appropriate Behavioral Paradigms	467
14.3.1.1	Reinforcer-Specific Controls	469
14.3.1.2	Neuroanatomical Controls	469
14.3.2	Role of CNS Circuitry in Mediating EtOH Self-Administration	470
14.3.3	Novel Neuroanatomical Substrates Regulating Alcohol Reward	471
14.4	Dopamine Neuronal Systems and Substrates Regulating Alcohol Reward	471
14.4.1	Dopaminergic Regulation in Alcohol Self-Administration: Review of Previous Research	472
14.5	Novel Dopamine Substrates Regulating Alcohol Reward	473
14.5.1	Bed Nucleus of Stria Terminalis	473
14.5.2	Ventral Pallidum	476
14.5.2.1	Hypothesized Mechanisms of Dopaminergic Regulation in Ventral Pallidum Regulating Alcohol-Motivated Behaviors	478
14.5.3	Lateral Hypothalamus	481
14.5.3.1	D ₂ Dopaminergic Regulation of Alcohol-Motivated Behaviors in LH: Hypothesized Mechanisms	481
14.5.4	Summary	485
14.6	Molecular Biology of the GABA _A BDZ Receptor	487
14.6.1	Commonalities of Alcohol and Modulators of the GABA _A BDZ Systems	487
14.6.2	GABA _A BDZ Modulation of EtOH Self-Administration: Studies Using Systematic Application of Probes	488
14.6.2.1	Significance of Systemic Studies	492

14.6.3	Site-Specific Microinjection Studies: Manipulation of GABA _A BDZ Receptor Complex in Modifying EtOH Self-Administration	493
14.6.4	Studies Supporting Hypothesis That GABA–DA Interactions Regulate Alcohol-Motivated Behaviors: GABAergic Modulation of DA Function	496
14.6.5	Conceptual Framework for Hypothesis Generation and Interpretation of GABA–DA Interaction in Alcohol Drinking Behavior	497
14.6.6	Novel CNS GABAergic Substrates Regulating Alcohol-Motivated Behaviors	498
14.6.7	Ligands with Preferential Selectivities for GABA _A Receptors Containing α_1 Subunit (GABA _{A1} Receptors)	498
14.6.8	Efficacies of β CCt and 3PBC in Modulating GABA Responses in Recombinant GABA _{A1,2,3,5} Receptors	499
14.7	Employing Ligands with Preferential Selectivities for α_1 Subunit Containing GABA _A Receptor as Pharmacological Probes to Investigate Novel Alcohol Reward Substrates	501
14.7.1	Ventral Pallidum	501
14.7.2	Systemic Studies	503
14.7.3	Microinjection Studies	505
14.8	Oral Administration of β CCt or 3PBC Produces Prolonged Reduction on Alcohol Self-Administration: Direct Comparison with Opioid Receptor Antagonist Naltrexone	507
14.8.1	Oral Efficacy of β CCt and 3PBC in Reducing Anxiety in Alcohol-Preferring Rats	510
14.9	Employing Ligands with Preferential Selectivities for α_5 -Subunit-Containing GABA _A Receptors as Pharmacological Probes to Investigate Novel Alcohol Reward Substrates: CA1 and CA3 Hippocampus	511
14.10	Subunit Selectivity versus Intrinsic Efficacy	516
14.11	Conclusions	517
	References	518

14.1 INTRODUCTION

Over the past two decades it has become increasingly clear that the disease of alcoholism, like other substance abuse addictions, is a brain disorder. Thus, one of the key objectives of alcoholism research is to understand the neuroanatomical substrates and neurocircuitry within the brain regulating alcohol addiction and dependence in humans. Unfortunately, alcoholism in humans is a complex disorder comprising not only central nervous system (CNS) mechanisms [1] but also social, cultural, and emotional influences [2]. Through basic neuroscience research, scientists in the alcohol field, particularly researchers focused on alcohol self-administration [3–9], are gaining a better understanding of the neuromechanism(s) regulating alcohol drinking behavior. This success has largely been attributable to the development and use of animal models [1].

14.2 OPTIMAL ANIMAL MODEL TO STUDY NEUROANATOMICAL SUBSTRATES OF ALCOHOL SELF-ADMINISTRATION BEHAVIORS

Animal models of alcoholism have provided useful analogs of the human condition, and a number of researchers have employed these models to study the neurobiological

factors mediating alcohol self-administration. As noted by Li (see [1], p. 36), such models are useful only “when they reveal some aspects of the complex process to yield understanding about the human condition.” Thus, to model the human condition, with specific considerations of criteria of alcoholism of the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV), genetically selective breeding of rats that initiate excessive alcohol drinking has produced an unequivocal impact in the field of alcoholism research. Exemplary of the genetically selected rodent is the alcohol-preferring (P) and high alcohol drinking (HAD) rat lines developed by Indiana University’s Alcohol Research Center. One of the primary criteria for an animal model of alcoholism [10], similar to the DSM-IV criterion in humans, is that individuals/rodents will work at high levels to obtain alcohol and that a great deal of time is spent in activities necessary to obtain alcohol. This criterion for alcoholism attests to the reinforcing strength/efficacy of alcohol as an abused substance. In comparison to most genetically selected rodent models of alcohol drinking, the efficacy profile of the Indiana selected rat models, particularly the P line in the operant paradigm, far exceeds that of the other rodent models (see [4, 11–13]). Thus, to model the human condition of alcohol abuse, in the present review, we have primarily selected as subjects the P rats, and the HAD rats to a lesser degree. Further, the P rat line has been shown to satisfy most of the criteria for an animal model of human alcohol abuse [10] to the satisfaction of the alcohol research community [8]. While the criteria have been discussed at length in the excellent review by McBride and Li [1], a brief summary of these findings is warranted to provide the reader with the rationale for using the P rat as subjects in the study of neurobiological mechanisms underlying alcohol self-administration behaviors. Other summaries in relation to the P rat line as an optimal model of the human condition of alcoholism/abuse can also be found in the literature [3, 8, 11]. Nevertheless, the P rat will: (1) voluntarily consume 5–8 g/kg of alcohol to attain blood alcohol concentrations of 50–200 mg%; (2) lever press for alcohol orally in concentrations of 10–40%, despite the fact that water and food are concurrently available; (3) drink alcohol for its pharmacological effect and not solely because of its taste, smell, or caloric properties as evidenced by self-administration of alcohol intragastrically and intracranially by P rats; (4) develop both metabolic and functional tolerance with free-choice alcohol drinking; and (5) develop physical dependence and signs of withdrawal following removal of alcohol after periods of chronic consumption. Taken together, the P rat line is among the best, albeit not perfect, models of human alcoholism [1].

14.3 INTEGRATING P RAT LINE WITH APPROPRIATE BEHAVIORAL PARADIGMS AND NEUROANATOMICAL STUDIES TO MAKE INFERENCES ABOUT NOVEL NEUROANATOMICAL SUBSTRATES OF ALCOHOL SELF-ADMINISTRATION BEHAVIORS

14.3.1 Appropriate Behavioral Paradigms

A variety of procedures have been successfully used to motivate rodents to orally self-administer alcohol [4]. The most widely used and accepted method in the alcohol field, particularly with outbred rats, is the sucrose-fading technique developed by Samson [14, 15]. Over the years, researchers have validated as well as modified this procedure for both limited (e.g., 10–60 min) and more prolonged exposure periods

(e.g., 2–4 h) [15, 16]. Modifications to the procedure wherein non-food- and water-deprived rats trained to initiate ethanol (EtOH) responding are allowed to obtain water or EtOH by responding at one of two levers have produced an operant choice paradigm that can be used to investigate the neuropharmacological bases of alcoholism [4, 17]. Other modifications of the procedure include a two-lever design wherein nondeprived rats lever press for alcohol on one lever and a highly palatable/isocaloric solution on the other (see [18, 19]). Some of the important features incorporated in this model include: (a) maintenance of responding for EtOH as a measure of EtOH reinforcement; (b) an index of preference for EtOH over water, independent of the absolute amounts of EtOH consumed; (c) a control for nonspecific drug effects on ingestive behaviors; (d) dissociation of the pharmacological motivation from consummatory processes; and (e) measurement of significant blood alcohol concentration (BAC) levels (see [4], also [17]).

In addition to the sucrose-fading technique, a number of laboratories have developed other procedures wherein pharmacologically relevant EtOH concentrations are obtained after training rats to orally ingest alcohol. Such techniques have been used in the home cage with both outbred rats [20–22] and rats selectively bred for alcohol consumption [1, 8]. For example, the ascending two-bottle water choice [20, 21] and the sweetened cocktail solution procedures [22] have been reported to produce EtOH intake levels in outbred rats that result in pharmacologically relevant EtOH concentrations. In addition, variations in the 24-h home cage model using limited-access procedures (e.g., 30–240 min) have been quite effective in producing significant alcohol levels and have been employed extensively in studying the neurobiological mechanisms of alcohol drinking, even in alcohol selectively bred rodents (Ps, HADs) [23–26]. Unfortunately, the investigator has little control over the contingent relations between responses and ingestion in this home cage model. Roberts and colleagues [27] suggested employing the home cage as the only reinforcing model “is potentially confounded by palatability.” As in studies of other abused drugs, the most appropriate instrument is the operant conditioning chamber where the contingency between responding and drinking can be specified and the volume of liquid ingested per completed schedule unit can be controlled. The operant methodology is especially important in analyzing complex dose–response relationships of a test agent [28, 29] and when examining the effects of a test agent on EtOH and another concurrently available reinforcer [5, 28, 30–33]. Finally, when the 24-h access model is employed, it is difficult to determine pharmacologically relevant BACs, since the scheduled drinking bout(s) is often difficult to ascertain. BACs are important when trying to determine the neuromechanism of action of EtOH [34–36]. Hence, the operant paradigm has a number of advantages over simple home cage drinking procedures in rats and mice [4, 27].

As noted above, the most widely used and accepted method of initiating operant responding for alcohol drinking in outbred and alcohol selected rats is the sucrose-fading technique [14]. The merits of this initiation procedure have been discussed in several previous reviews [1, 4]. However, while a number of laboratories are currently evaluating the efficacy of pharmacological treatments on scheduled controlled responding using the Samson procedure, studies in which specific controls are used for rate dependency and the postingestional properties of the test solutions are generally lacking in the alcohol literature. As a result, many published reports are very difficult to interpret in relation to the selectivity of a test agent reducing alcohol

drinking on the one hand, and the degree to which inferences can be made from these studies to neural mechanisms of action on the other. It is important to note that selectivity for alcohol drinking is a very important concern in self-administration studies given that most studies in the literature report that test compounds “all” reduce motivated responding for alcohol. Elevations in alcohol-motivated behaviors, particularly within the operant chamber in non-food- or water-deprived rodents, have generally not been reported in the alcohol literature. Thus, to eliminate confounding of results/interpretation, it is important to use *rigorous reinforcer control procedures* in alcohol self-administration research. While a thorough review of these procedures is beyond the scope of this chapter, below we provide a brief review of protocols that can be used to optimize data interpretation in rodent alcohol-self-administration research. For more details on these procedures, the interested reader should consult the protocols outlined in June [4].

14.3.1.1 Reinforcer-Specific Controls. First, to investigate the capacity of a pharmacological agent to produce dose and time course effects when rats are presented with EtOH as the sole reinforcer, rodents should be given the EtOH solution on a fixed-ratio (FR) schedule, preferably an FR4 or FR6. Second, the capacity of a pharmacological agent to produce dose and time course effects when rats are presented with a palatable reinforcer (e.g., saccharin, sucrose) as the sole reinforcer on an FR schedule should also be determined. When the data are interpreted, one is able to make inferences as to whether antagonism is specific to EtOH-motivated behaviors. Third, the researcher should evaluate the capacity of a pharmacological agent to produce dose and time course effects when a concurrent schedule procedure is employed under an FR schedule [37]. Because basal response rates should be equal or near equal between groups in this protocol, the concurrent schedule will address the issue of whether the capacity of an agent to decrease EtOH and not saccharin reinforcement is due to differences in reinforcing efficacy (i.e., strength). Finally, the capacity of a pharmacological agent to produce dose and time course effects under a concurrent FR schedule procedure when two isocaloric alternative solutions are presented should be evaluated. The caloric consideration is important insofar as the calories contained in alcohol could contribute substantially to its motivating properties [32], independent of the CNS pharmacological effects. It should also be noted that Bodnar and his colleagues [38, 39] have reported a differential regulation of caloric compared with noncaloric reinforcers within the opioid systems. Thus, the CNS substrates which regulate a particular type of palatable ingesta may be different. It is well established that an isocaloric concurrent procedure for alcohol and the alternative solution may produce different response rates and profiles (see [40]).

14.3.1.2 Neuroanatomical Controls. Although reinforcer-specific controls add immensely in making inferences about pharmacological probes in investigating neuroanatomical substrates of alcohol-motivated behaviors, equally important is the use of neuroanatomical controls. Such studies are rarely performed in published reports in the alcohol field. CNS substrates are primarily studied in alcohol research using microinjection procedures (i.e., intracerebral drug delivery) [1, 9]. Generally, there are two types of neuroanatomical control groups that will assist in data interpretation (see [4]). First, a researcher may use a “region-specific” control wherein a second infusion is performed 1–2 mm away from the designated brain site. The second

infusion may be given rostral, caudal, ventral, or dorsal to the designated brain site. Studies that attempt to compare the anterior and posterior ventral tegmental area (VTA) or the nucleus accumbens (NAcc) shell and core or the NAcc and ventral striatum are also examples of “region-specific” comparisons. For illustrative examples of such control procedures, studies by Nowak et al. [41] and June et al. [19, 29] should be reviewed. Alternatively, a researcher may also use an entirely different locus as the control site. This type of control is referred to as an “alternate region” control. The alternate region control is particularly important when different receptor populations or subunit configurations are of interest. Such controls are illustrated in the work by Eiler et al. [42] wherein the caudate putamen was used as an alternative locus in investigating the dorsal bed nucleus of the stria terminalis. Harvey et al. [37] also used the alternate region design wherein the NAcc and caudate putamen were used as control loci in investigating the ventral pallidum, a putative drug reward substrate ventral to the NAcc [43].

In summary, this section has provided information pertaining to the P rat as a model of alcoholism. The model has a number of advantages over other currently available models, and with the exception of variables relating to psychosocial and cultural factors which influence drinking, the P rat satisfies the DSM-IV criteria reasonably well for the condition of alcoholism in humans [1]. Besides having an “optimal animal model,” both reinforcer (i.e., pertaining to ingesta) and neuroanatomical control procedures were discussed and suggested to be critical in making direct inferences about putative neuromechanisms of alcohol-motivated behaviors. We propose that integration of the ideal animal model combined with systematic utilization of reinforcer and neuroanatomical specific control designs is a multidisciplinary approach which provides a researcher with a wealth of data for systematic evaluation and interpretation of pharmacological probes to understand the neuromechanisms regulating alcohol-motivated behaviors.

14.3.2 Role of CNS Circuitry in Mediating EtOH Self-Administration

An understanding of the neuroanatomical substrates, neurocircuitry, and interaction of the neuronal systems which regulate alcohol drinking is key in the identification of novel targets for drugs to treat alcohol addiction and dependence in humans [19, 37, 44]. While alcohol affects an array of neuronal systems, including opioids, glutamate, γ -aminobutyric acid (GABA), adenosine, serotonin, and the catecholamines [1, 45–47], alcohol also shares common neural substrates, circuitry, and characteristics with other addictive substances. Specifically, alcohol shares the property of increasing dopaminergic activity in the mesocorticolimbic system [48]. Within the mesocorticolimbic system, previous research focused on the VTA of the midbrain and its projection to the limbic forebrain, the NAcc (for a review see [46]). Indeed, prior work in animal models and humans provides evidence that all drugs of abuse converge on this pathway to produce their reinforcing effects ([49]; for a recent review see [50]). However, as discussed in a recent review by Lovinger and Crabbe [44], alcohol abuse and alcoholism have a number of distinct neuronal processes from other substance abuse disorders. For example, recent work has demonstrated that a number of additional brain areas independently or in collaboration with the VTA and NAcc play an important role in regulating alcohol drinking behaviors [46, 51, 52]. Specifically, regions of the extended amygdala circuit [central nucleus of the

amygdala, bed nucleus of the stria terminalis, shell of the NAcc, sublenticular substantia innominata (SI)/ventral pallidum] [53, 54], hippocampus, and lateral hypothalamus have all been shown to regulate alcohol drinking ([30, 42, 55, 56]; also for a review see [19]). As noted by Nestler [50], because some of these areas are associated with the traditional memory system, the study of these new substrates could provide information about the cognitive factors and emotional memories which might regulate alcohol drinking and relapse behavior. In addition, because brain areas such as the hypothalamus may play a role in alcohol addiction as well as the so-called natural addictions (e.g., obesity, sex) [57, 58], these areas could provide strategies to understand the common/shared neuronal pathways which regulate the behavioral pathology of alcohol and other consummatory behaviors.

14.3.3 Novel Neuroanatomical Substrates Regulating Alcohol Reward

Research on the substrates and circuitry regulating alcohol self-administration has experienced an enormous growth over the past decade. Several reviews have described this area of inquiry [1, 8, 9, 59]. In many cases, these reviews have provided an exhaustive coverage of the neuronal substrates and systems which have been shown to regulate alcohol-motivated behaviors. In the present review, we will focus exclusively on relatively new findings in the alcohol self-administration literature (many of which were not available or omitted in prior reviews), implicating novel substrates and circuitry within the dopaminergic and GABAergic systems. The *majority* of the recent studies described here have employed rigorous reinforcer and neuroanatomical specific control designs, allowing for more precise inferences about putative alcohol reward mechanisms. The focus on the dopamine (DA) and GABA systems relates to both neuroanatomical topography and established neurochemistry, demonstrating that within several loci in the CNS a neuromodulatory role may exist between the two systems. New findings will be presented demonstrating that exploitation of the GABAergic system indeed represents a vital strategic route for the development of novel prototype ligands to reduce alcohol drinking behavior in human alcoholics. Moreover, we propose that GABA mediates its actions on alcohol self-administration via interaction with the DA system [19, 37]. Hence, in this regard, a review of the literature pertaining to DAergic regulatory control on alcohol self-administration is warranted. Finally, it is clear that a number of other important neuronal systems have been shown to be important in regulating alcohol reward mechanisms, particularly imbalances in corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) [60–63]. A discussion of this literature is beyond the scope of the present review.

14.4 DOPAMINE NEURONAL SYSTEMS AND SUBSTRATES REGULATING ALCOHOL REWARD

According to the DA hypothesis of reward, DA systems directly mediate the rewarding or primary motivational characteristics of natural stimuli such as food, water, and sex as well as various drugs of abuse, including EtOH [57]. The most current research examining the role of DA in the reinforcing properties of EtOH has focused on the interactions of the NAcc and the A10 DA cell body grouping known

as the VTA (for a review see [51]). The NAcc receives its extensive DA innervation from the VTA. This DAergic fiber tract is referred to as the mesolimbic pathway (for a review see [64]). Below, we provide a selective review of the early systemic, site-specific microinjection and lesion studies that have been critical in providing support for a dopaminergic regulation in alcohol self-administration research.

14.4.1 Dopaminergic Regulation in Alcohol Self-Administration: Review of Previous Research

A body of research employing systemic injections has implicated DA neurotransmission in alcohol reinforcement. Generally, this research has demonstrated that neurochemical manipulations which increase DA transmission increase EtOH intake and responding, while manipulations that reduce DA transmission reduce responding and intake; however, conflicting reports have emerged (for a review see [1, 9, 17, 51]). Initial studies employing site-specific microinjection methodology evaluated the effects of bilateral infusions of the D₁ antagonist SCH 23390 (0.1–2.0 µg/side) and the D₂ antagonist sulpiride (0.1–2.0 µg/side) into the NAcc of P rats during a 30-min home cage EtOH access period [65]. The D₂ antagonist sulpiride produced a significant dose-dependent increase in alcohol drinking, while no significant effects were seen with SCH 23390. Subsequent studies by Samson and his colleagues [9, 66, 67] have shown that bilateral intra-accumbens infusions of the nonspecific agonist *d*-amphetamine (4–20 µg) or the more selective DA D₂-like (i.e., selectivity toward D₂ compared to D₃ and D₄) agonist quinpirole (1 µg) increased EtOH responding in outbred rats. In this same study, however, higher quinpirole doses (4 and 10 µg) significantly decreased EtOH responding. Samson and his colleagues have also shown that reducing DA neurotransmission in the NAcc directly by local administration of the D₂-like antagonist raclopride [9] or indirectly by infusion of the D₂ agonist quinpirole [66] into the VTA decreased EtOH responding. It should be noted that quinpirole in the VTA inhibits DA cell activity via a proposed feedback of the cell upon itself [68]; hence, it would be predicted that quinpirole would produce effects similar to blockade of DA neurotransmission in the terminal field by raclopride. Indeed, Murphy and his colleagues (see [41]) demonstrated that direct infusion of quinpirole as well as another D₂ agonist quinlorane in the VTA of P rats reduces home cage alcohol but not saccharin drinking. It should be noted that a selective reduction on alcohol was observed in the anterior, but not the posterior, VTA, suggesting that only those D₂ receptors on cell bodies within the anterior VTA selectively regulate alcohol intake [8]. One hypothesis that has been used to explain the effectiveness of DA agonists in decreasing EtOH self-administration is that agonists blunt the reinforcing actions of EtOH by substituting for the DA-enhancing action of the agent. The heightened “hedonic state” produced by DA receptor activation may eliminate the rats’ motivation to respond for EtOH [69, 70].

In addition to the pharmacological microinjection studies discussed above, substantial neurochemical evidence supports a role of the mesoaccumbens DA system in mediating EtOH-motivated behaviors [71–73]. These relatively recent neurochemical studies are discussed below. However, not all studies support a direct role for the DA system in alcohol self-administration. Specifically, early neurobehavioral studies with neurotoxic lesions [74–76] suggested that norepinephrine (NE) may play a more important role than DA in EtOH self-administration. Pharmacological manipulations

using highly selective DA receptor agents have also failed to alter EtOH self-administration [77]. Nonetheless, it should be noted that these studies used systemic administration, not examining discrete brain loci. Previous work by Rassnick et al. [78] have shown that 6-Hydroxy-Dopamine (6-OHDA) lesions of the NAcc do not block established oral EtOH-reinforced responding on a continuous reinforcement schedule. Rassnick and her colleagues have interpreted these data to mean that the mesoaccumbens pathway is not the only system involved in mediating EtOH reinforcement. Research involving the infusion of DA agonists and antagonists into the NAcc is also equivocal. For example, while Samson and his colleagues demonstrated that DA antagonists infused in the NAcc decrease EtOH-reinforced responding [67], dose-dependent increases were observed by Levy et al. [65] with DA antagonists. While this discrepancy has not been resolved in the current literature, one possible explanation to account for these findings is that altered responses to DA blockade in P rats might result from neurobiological changes in DA systems and cause P rats to initiate rather than suppress responding/intake [1].

14.5 NOVEL DOPAMINE SUBSTRATES REGULATING ALCOHOL REWARD

More recent research has begun to focus on brain substrates other than the NAcc that are innervated by the VTA. Many of these loci have been shown to play a role in mediating the rewarding properties of multiple drugs of abuse [79]. This section examines recent evidence that implicates these new loci in the regulation of EtOH reward. For each locus reviewed, the effects of EtOH on the DA system within the substrate will be discussed, as will any effect DA manipulation has on the mediation of EtOH reinforcement. The first substrate to be discussed is the bed nucleus of the stria terminalis (BST) a forebrain structure innervated by DA within the VTA [80]. The second structure examined is the ventral pallidum (VP), a ventral forebrain structure that receives dopaminergic input from the VTA via the mesopallidal pathway [81, 82]. The final substrate, the lateral hypothalamus (LH), is best known for its control of feeding and drinking behavior [83–85]. It receives innervation from DA neurons originating within the VTA via the mesothalamic pathway [64, 86].

14.5.1 Bed Nucleus of Stria Terminalis

Recent evidence has emerged suggesting that the BST exerts dopaminergic control over the reinforcing properties of EtOH [42, 48]. The BST is perhaps best known as a component of the extended amygdala (EA), a group of forebrain nuclei that exhibit similar morphology, immunoreactivity, and connectivity (including the NAcc shell, central nucleus of the amygdala, and substantia innominata) [53]. As with other members of the EA, the BST receives extensive dopaminergic innervation from the DA cell bodies located within the VTA via the mesolimbic path [87, 88]. DA input from the VTA is received within the BST by a 3:2 ratio of D₁-like to D₂-like DA receptors [80, 89, 90]. Within the BST, several other neurotransmitters (e.g., GABA, glutamate, and various opioid peptides) also facilitate neuronal communication to the other putative alcohol reward substrates of the extended amygdala, such as the NAcc and central nucleus of the amygdala (CeA), as well as other reward areas outside the

EA, such as the VP and the hippocampus [80, 87, 88, 91–93]. The BST has also been found to send GABAergic connections back to the VTA, perhaps to regulate the VTA dopaminergic output [94, 95].

The first study examining the role of the dopaminergic system within the BST on acute EtOH administration was conducted by Carboni and colleagues in 2000 [48]. In this study, Carboni et al. examined DA release in the BST following peripheral administration of various drugs of abuse. To accomplish this, a microdialysis probe was implanted in the anterior BST of male Sprague–Dawley rats. After the recovery phase, the rats were given systemic cocaine, the selective DA reuptake inhibitor GBR12909, morphine, nicotine, and alcohol (0.25 and 0.5 g/kg) (see Fig. 14.1). Their data demonstrated that all drugs led to significant increases in extracellular DA in the BST, lending increased support for a role of the BST in the mediation of drug reward. It should be noted that the 0.25- and 0.5-g/kg alcohol doses represent levels that are obtained during alcohol responding and intake [4]. In addition, these doses typically induce locomotor activation in rodents [96, 97], particularly during the ascending limb of the BAC [96]. Hence, to the extent that the locomotor activational effects in rodents may be a putative model of alcohol-induced euphoria in humans [97, 98], the findings by Carboni and colleagues could have relevance to alcohol's rewarding properties. Nevertheless, Carboni and colleagues [48] suggested that the EtOH-induced increase of DA in the BST might be analogous to the DA release in the NAcc.

While the Carboni et al. study was the first to suggest the DA system within the BST may be involved in EtOH reward, this study obviously does not directly test the

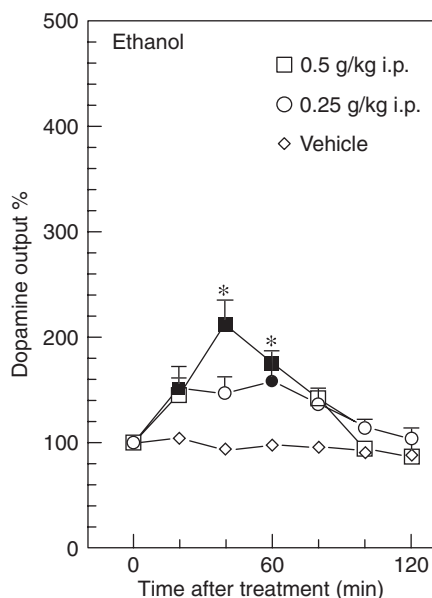


Figure 14.1 Effect of ethanol following intraperitoneal injection of 0.25 and 0.5 g/kg (injected as 10% v/v solution) on dopamine concentration in dialysate obtained by in vivo microdialysis from BST. Each point is the mean [standard error of the mean (SEM)] of at least four determinations. Filled symbols: p , 0.05 from basal values concentration; (*) p , 0.05 from corresponding time point of vehicle group. Adapted from [48].

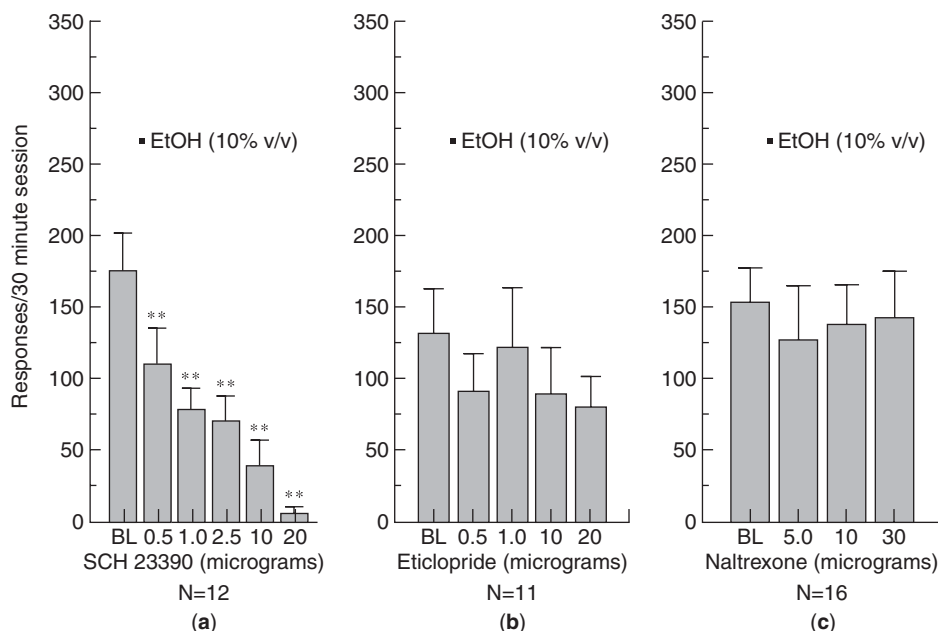


Figure 14.2 (a) Effects of SCH 23390 (0.5–20 µg) following bilateral injection in bed nucleus of stria terminalis (BST) on responding maintained by EtOH (10% v/v). Data are shown as mean \pm SEM. (**) $p \leq 0.01$ SCH 23390 vs. baseline (noninfusion and saline infusion controls). (b) Effects of eticlopride (0.5–20 µg) following bilateral injection in BST on responding maintained by EtOH (10% v/v). Data are mean \pm SEM. Adapted from Eiler et al., 2003.

hypothesis. Eiler et al. [42] directly investigated the potential role of DA (in the BST) on EtOH reinforcement using the microinfusion technique. To accomplish this, P rats were bilaterally infused with the D_1 receptor antagonist SCH 23390 or D_2 -like receptor antagonist eticlopride in the dorsolateral BST following training to lever press for EtOH in an operant paradigm. The results were clear-cut. SCH 23390 produced significant dose reductions on responding maintained by alcohol; however, eticlopride and naltrexone were not effective (Fig. 14.2). These data strongly suggest that EtOH reward is mediated by D_1 -like receptors located within the BST. As EtOH consumption at its core is a consumptive, drinking behavior, it is important to determine if the effects of any drug on EtOH are selective or a result of a suppression of drinking behavior in general. To evaluate reinforcer specificity, animals were trained to operantly self-administer sucrose. In contrast to the findings seen with EtOH, the two highest SCH 23390 and eticlopride doses (2.5 and 20.0 µg) significantly reduced sucrose responding; however, naltrexone was not effective. The results of this study were the first to demonstrate that a D_1 DA receptor subtype within the BST plays a significant role in EtOH reinforcement. While the D_1 receptors also appear to have a regulatory role in sucrose reinforcement, the BST appears most sensitive to EtOH reinforcement. In contrast, neither D_2 receptors nor the opioid system plays a role in the regulation of alcohol-rewarding properties in the BST. Similarly, the opioid systems of the BST do not appear significant in mediating the rewarding properties of sweet palatable solutions.

14.5.2 Ventral Pallidum

Increasing evidence implicates the VP as a dopaminoreceptive brain region regulating drug reinforcement [99–101]. More recent studies, however, have evaluated the ventral pallidal DA system in regulating alcohol-motivated behaviors. One of the first studies to investigate the role of the VP in EtOH reward was conducted by Melendez and colleagues [102]. This study evaluated the levels of extracellular DA within the VP following intraperitoneal administrations of EtOH. Two groups of Wistar rats were surgically implanted with bilateral microdialysis probes aimed at either the VP and the globus pallidus (GP) or the NAcc and the dorsal striatum (dSTR). EtOH (0.0–2.25 g/kg) produced a dose-dependent increase in extracellular DA in the VP but not the GP, with increases similar to those seen in the VP observed in both the NAcc and the dSTR following a 2.25-g/kg dose of EtOH (Fig. 14.3).

Melendez et al. [102] concluded that these data indicated that the mesopallidal system is more sensitive to the effects of EtOH than the nigropallidal. These data, together with results of the NAcc/dSTR portion of the study, suggest that the ventral regions of the striatopallidal complex are more sensitive to the effects of EtOH than the dorsal aspect. The sensitivity of these ventral circuits is in agreement with research that indicates this circuitry is important in mediating the rewarding effects of various drugs of abuse, including EtOH. Unfortunately, however, unlike the Carboni et al. study [48], the alcohol doses used in this study are exceptionally high, and it is not clear what their relevance to alcohol reward might be. As a result, these data should be interpreted with caution when making inferences to alcohol-rewarding effects.

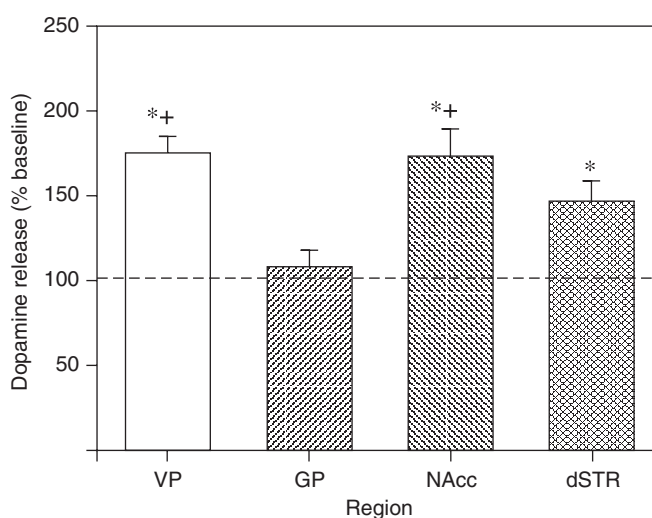


Figure 14.3 Time course effect of sterile saline or 15% (v/v) ethanol at doses of 0.75, 1.5, or 2.25 g/kg on extracellular levels of dopamine in VP and GP of Wistar rats. Values are expressed as percentage of baseline values and represent mean \pm SEM. The i.p. administration of saline or 0.75 g/kg failed to significantly alter the extracellular levels of dopamine in either the VP or the GP ($p > 0.05$). (*) $p < 0.05$ as compared to saline (Dunnett's t); (+) $p < 0.05$ as compared to the GP [one-way analysis of variance (ANOVA)]. Adapted from Melendez et al., 2003.

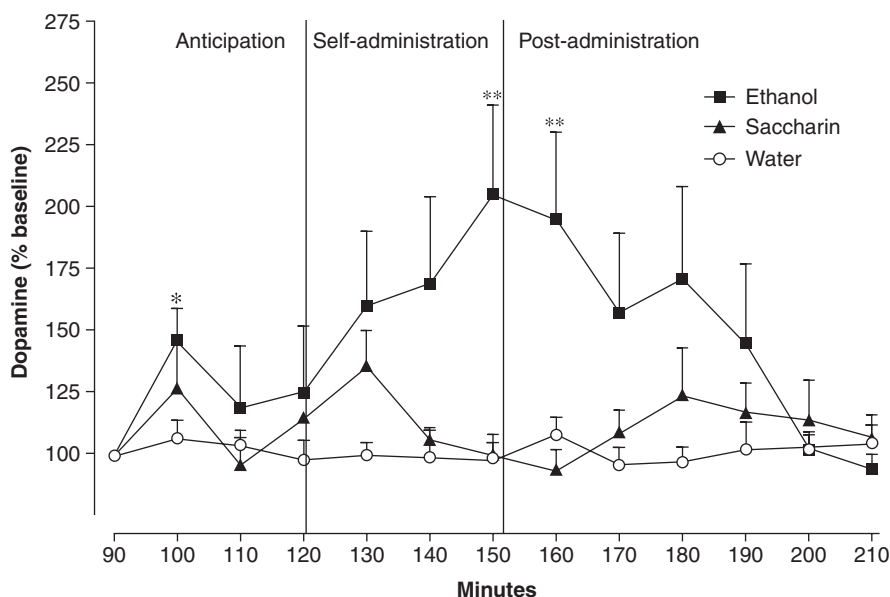


Figure 14.4 Extracellular levels of dopamine in VP of ethanol, saccharin, and water (control) groups expressed as percentage of baseline during anticipation, self-administration, and postadministration periods. Data are means \pm SEM. Baseline dialysate levels of dopamine of ethanol, saccharin, and water groups were not significantly different (1.1 ± 0.1 , 0.93 ± 0.2 , and 0.85 ± 0.1 nM, respectively). (*) $p < 0.05$ compared with values for water; (**) $p < 0.05$ compared with values for water and saccharin with posthoc Tukey b test. Adapted from Melendez et al., 2004.

In 2004, Melendez and colleagues continued examining the role of the DA system within the VP on EtOH reward [56]. Female P rats, implanted with guide cannula into the VP, operantly self-administered EtOH, water, or saccharin during a microdialysis session divided into four discrete periods: habituation, anticipation, self-administration, and postadministration. While self-administration of saccharin or water had no effect on DA levels within the VP, the presentation of EtOH led to a marked increase in DA output in the anticipatory phase as well as increases throughout the self-administration phase, which decreased postadministration (see Fig. 14.4). Together, the data revealed that, as with intraperitoneal (i.p.) administration, self-administration of EtOH also led to significant increases in extracellular DA levels within the VP. These data further suggest a role of the VP in the anticipation of EtOH reward as well.

In their most recent study, Melendez and colleagues [26] assessed the effect of D_1 and D_2 DA receptor blockade within the VP on the intake of EtOH in a limited-access home cage paradigm employing similar extracellular DA measurement methods. P rats were separated into two groups and implanted with either bilateral guide cannula in the VP or a unilateral microdialysis probe aimed at the VP. Rats of the microinfusion group initiated EtOH intake during a 60-min limited-access paradigm following the microinjection of either the D_1 antagonist SCH 23390 or the D_2 antagonist sulpiride. Animals in the microdialysis group were evaluated for increases in extracellular DA levels following the reverse dialysis of either SCH 23390 or sulpiride.

In the limited-access/microinfusion portion of the experiment, only the highest tested dose of sulpiride (2.0 μg) was effective, producing an increase in intake. Interestingly, while only the highest dose of sulpiride (200 μM) led to an increase in extracellular DA with the VP, all tested doses (10–200 μM) of SCH 23390 were effective.

The data from the limited-access portion of this study suggest that D_2 receptors within the VP (that may be located on GABAergic neurons) play an important role in the regulation of EtOH reward. Evidence exists that such neurons exit from the VP and innervate the NAcc [103]. It is possible that the blockade of these receptors disinhibit the NAcc or perhaps other reward loci innervated by GABA neurons originating from within the VP. Data from the microdialysis portion of this study seem to support the hypothesis insofar as the minimal increase in extracellular DA suggests that the D_2 receptors within the VP are not acting as autoreceptors. This further strengthens the theory that the increase in intake following D_2 blockade is accomplished by non-dopaminergic neurons. Unlike the D_2 system, the D_1 receptor system within the VP appears to play only a minimal role in the regulation of EtOH reward. Reverse microdialysis of SCH 23390, however, led to significant increases in DA levels within the VP. This finding seems to imply the DA release within the VP is regulated in large part by a yet-undetermined D_1 receptor-mediated inhibitory feedback loop. These data also suggest that while EtOH is capable of increasing extracellular DA within the VP, such an increase fails to alter EtOH intake.

Recently, our laboratory also evaluated the effects of DA receptor blockade within the VP on the self-administration of EtOH [104]. For this study, P rats were implanted with bilateral guide cannula aimed at the VP and trained to operantly self-administer EtOH. SCH 23390 (1.0–40.0 μg) and the D_2 -like selective receptor antagonist eticlopride (1.0–40.0 μg) were used for DA receptor blockade. The two highest doses of SCH 23390 (10.0, 40.0 μg) resulted in a marked reduction in lever pressing (Fig. 14.5a). These data implicate the D_1 receptor subtype in the mediation of EtOH, a finding that is somewhat contradictory to that seen in the 2005 Melendez study [26]. Similar to D_1 blockade, eticlopride also led to a reduction in EtOH responding when administered in high doses (20.0–40.0 μg) (Fig. 14.5b). However, when the 10.0- μg dose of eticlopride was microinfused into the VP, it produced a 41% increase in EtOH responding similar to the increases observed by Melendez and colleagues [26]. The overall findings from the research detailed above strongly implicate the VP as an important substrate in the regulation of EtOH self-administration. Microdialysis studies clearly demonstrate that EtOH, whether given via either i.p. injection or self-administration, readily increases DA release within the VP in a manner similar to that seen within the NAcc following exposure to EtOH (see [73]).

14.5.2.1 Hypothesized Mechanisms of Dopaminergic Regulation in Ventral Pallidum Regulating Alcohol-Motivated Behaviors. The precise mechanism(s) responsible for the increase in extracellular DA following EtOH are not clear. Several, hypotheses to explain this phenomenon have been proposed. Melendez et al. [102] suggested that the increased levels of DA within the VP may be higher than those of the GP due to differential sensitivity of the origins of the DA neurons that innervate them. In fact, the DA neurons within the VTA (which contains the cell bodies of the VP DA neurons) have been shown to be five times more sensitive to the effects of EtOH than

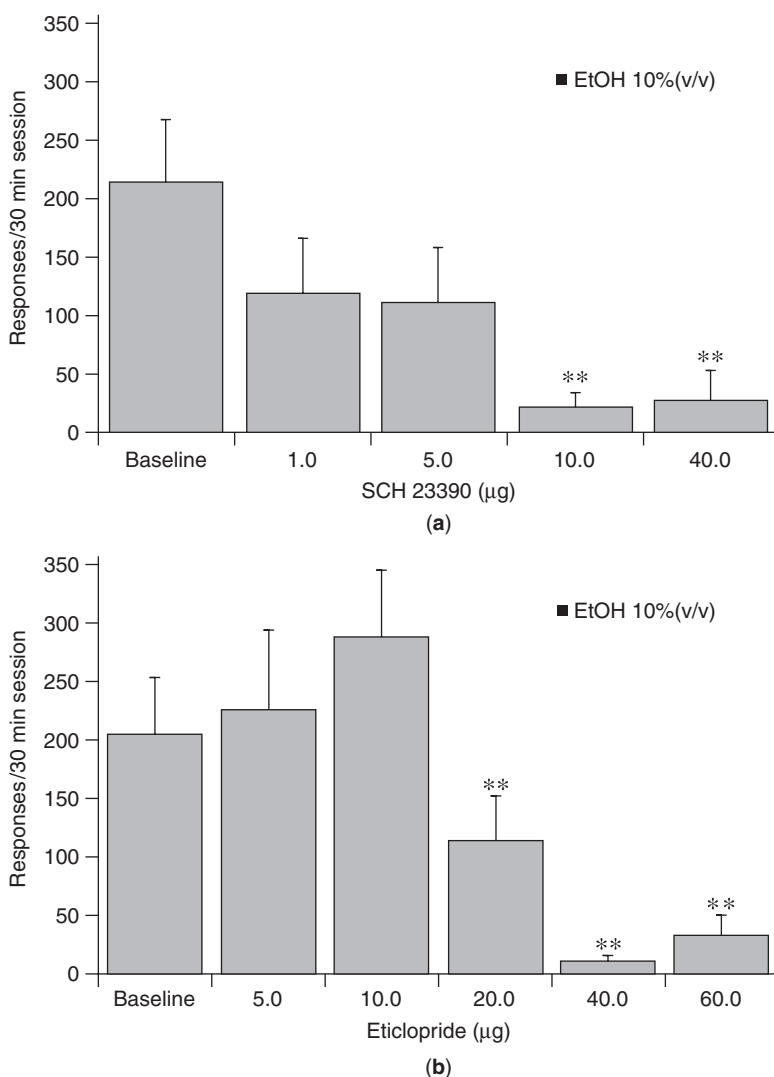


Figure 14.5 (a) Effects of SCH 23390 (1.0–40.0 μg) following bilateral injection in VP on responding maintained by EtOH (10% v/v). Data shown as mean ± SEM. (**) $p \leq 0.01$ SCH 23390 vs. baseline (noninfusion and saline infusion controls). (b) Effects of eticlopride (5.0–60.0 μg) following bilateral injection in VP on responding maintained by EtOH (10% v/v). Data shown as mean ± SEM. Adapted from Foster et al., 2002.

those found in the substantia nigra (SN), which innervates the GP [105]. The sensitivity of these neurons may ultimately be the result of GABAergic inhibition which is higher in the SN. It is also proposed that the differential sensitivity may be due to effects EtOH may have in the terminal fields, that is, that innervation from the striatum may affect the release of DA within the VP and GP. The majority of the striatopallidal neurons are GABAergic, and EtOH may act upon these neurons to regulate DA release. Blockade of both GABA_A and GABA_B receptors within the VP

results in increases in extracellular DA within the VP [106]. Further, June and colleagues [19, 37] have previously established a role of GABA_A receptors within the VP in the mediation of EtOH reward. It is also possible that a combination of these two factors could regulate EtOH-related VP DA increases.

The increases in EtOH intake observed by Melendez et al. [26] following D₂ blockade within the VP are compatible with the results seen in other studies involving the self-administration of EtOH [65], amphetamine [107], and cocaine [108]. It is hypothesized that the increase in responding observed is compensatory. Since blockade of the D₂ system may lower the reward efficacy of EtOH, in response the animals self-administer at higher rates in order to compensate for the reduced reinforcement. This hypothesis may be true for a partial blockade produced by lower doses of a D₂ antagonist; however, higher doses lead not to an increase in self-administration but to a decrease. It is possible that as more D₂ receptors are occupied, the reward value of self-administered EtOH reduces even further. While a partial blockade may result in compensatory increases in responding, a more complete blockade may produce such a robust reduction in reward value that the animals no longer possess the necessary motivation to self-administer the drug. As a potential mechanism, Melendez et al. [26] suggest that the D₂ receptors of the VP may reside presynaptically on efferent GABA_A neurons. However, the neuromechanisms regulating these effects are not clearly understood.

Effects on EtOH self-administration produced by the D₁ antagonist SCH 23390 have led to contradictory findings. For example, Melendez [26] reported that while SCH 23390 produced a robust increase in extracellular DA levels within the VP, it had no effect on EtOH intake. However, the study conducted by Foster et al. [104] found that SCH 23390 produced profound reductions in EtOH responding. Melendez suggests that while D₁ blockade does produce profound elevations in DA release within the VP, these increases are not sufficient to influence EtOH drinking behavior. Similar increases within the NAcc also produce no change in EtOH intake [109]. While the DA increase seen following the doses administered in the Melendez study may be insufficient to influence EtOH self-administration, it may be possible that the higher doses used in the Foster study could produce DA levels within the VP that are elevated so that they begin to substitute for the EtOH, thus reducing responding. It is also important to note that the cellular localization of the D₁ receptors within the VP has yet to be determined. It is therefore possible that at higher doses a D₁ antagonist could activate or inhibit pathways that result in the reduction of EtOH reward through a variety of means while still producing increases in extracellular DA within the VP.

Research indicates that the mediation of EtOH reward clearly involves the DA system within the VP. Not only is EtOH capable of producing increases in extracellular DA release within the VP, it also produces anticipatory effects within the VP. Furthermore, both D₁ and D₂ DA receptor subtypes within the VP appear to regulate the reinforcing properties of EtOH, with the D₁ receptors producing a decrease in EtOH self-administration while the D₂ receptors produce biphasic effects with low doses leading to increases and higher doses nearly abolishing EtOH self-administration. Together, the studies presented above suggest that both the D₁ and D₂ receptor subtypes are implicated in the regulation of EtOH self-administration; however, it is clear that while their regulatory control within the VP may be overlapping, there are distinct regulators within the VP which differentiate the two receptors.

14.5.3 Lateral Hypothalamus

The LH, like other reward substrates, receives dopaminergic innervation directly from DA cell bodies located within the VTA as part of the mesencephalic pathway [86, 110, 111]. The LH also contains a number of DA receptors with a higher ratio of D₂ to D₁ receptors [112]. Historically, the DA system within the LH has been implicated in the inhibitory control of both food and water intake [84, 85, 113, 114] as well as inhibiting the locomotor processes associated with the procurement of food and water [115]. This tonic inhibition can be easily removed by local administration of the selective D₂-like antagonist sulpiride, leading to increases in feeding and drinking as well as psychomotor activation [114–116]. The increase in these behaviors, particularly the psychomotor activation, suggests that the LH is in some way involved in mediating the rewarding aspects of eating and drinking along with the initiation of consumptive behaviors that precede intake, as there is often a link sited between the increase in locomotor activity and drug reinforcement [117].

Reward is often associated with the increased release of DA, particularly in brain regions associated with the regulation of the reinforcing properties of various drugs of abuse. Thus, any area that may mediate release of DA into reward areas such as the NAcc may also play a role in the regulation of drug reinforcement. The LH may be one such area. Early studies revealed that electrical stimulation of the LH leads to increased levels of DA and its metabolites within the NAcc [83]. To further investigate the LH's control of DA release within the NAcc, Parada and colleagues [118] examined the effects the D₂ antagonist sulpiride on the DA release within the NAcc. This study found that sulpiride injected directly into the LH led to a dose-dependent increase in DA release within the NAcc at the highest tested dose (Fig. 14.6). Moreover, this study demonstrated that the Sprague–Dawley rats used would lever press in an operant paradigm for direct infusion of sulpiride into the LH, demonstrating a strong link between increased DA release with the NAcc and the reward value of the D₂ antagonist sulpiride (Fig. 14.7). While the data above suggest that the LH is a prime candidate for a DA-mediated reward locus, research into its role in drug reward has been very limited.

June and his colleagues have examined the role of DA within the LH on the regulation of EtOH reward [119]. In this study, the effects of DA blockade using the D₁ antagonist SCH 23390 and the D₂-like receptor antagonist eticlopride were evaluated following bilateral infusion into the LH of P rats. Reinforcer specificity was determined by evaluation of the antagonist on sucrose-motivated responding. Neuroanatomical specificity in modulating alcohol- and sucrose-motivated behaviors was examined following bilateral injections of both SCH 23390 and eticlopride into the ventral thalamus. Figure 14.8b shows that eticlopride (1–40.0 µg) produced both a dose-related and profound reduction on responding maintained by alcohol. In contrast, Figure 14.8a shows that SCH 23390, the highly selective D₁ antagonist, did not alter alcohol responding (not shown are doses as high as 60 µg). Neither antagonist altered sucrose-maintained responding. Hence, eticlopride produces both reinforcer and neuroanatomical specificity in reducing alcohol drinking behaviors.

14.5.3.1 D₂ Dopaminergic Regulation of Alcohol-Motivated Behaviors in LH: Hypothesized Mechanisms. The above findings provide compelling data suggesting that blockade of D₂ receptors within the LH leads to a profound reduction in the

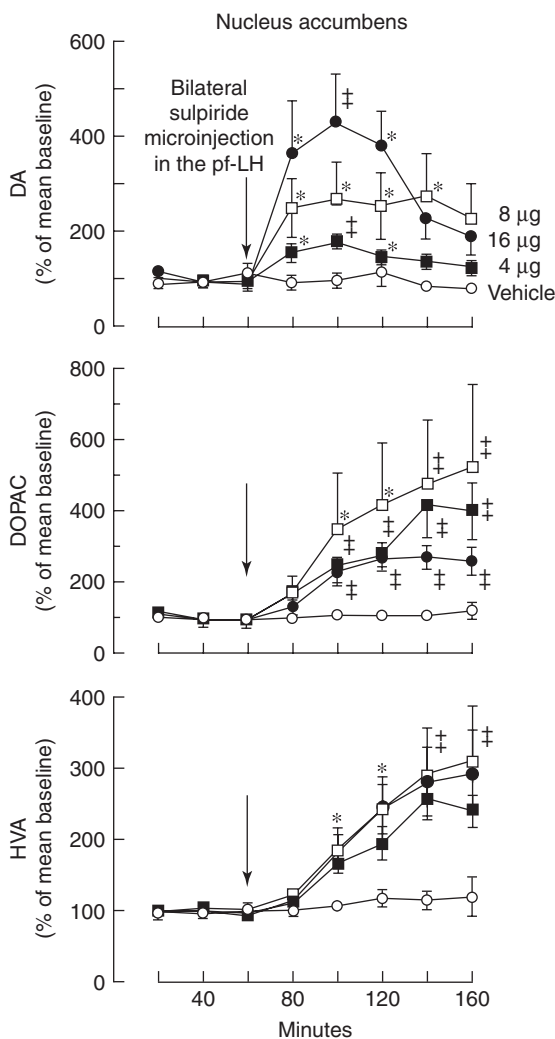


Figure 14.6 Sulpiride injected bilaterally in the prefrontal lateral hypothalamus (pf-LH) increases extracellular DA in NAcc. Curves show extracellular levels of DA (top graph), dihydroxyphenylacetic acid (DOPAC, middle graph), and HVA (bottom graph) in the right NAcc of rats receiving bilateral pf-LH microinjections of sulpiride (filled squares, 4 µg/0.3 µL; open squares, 8 µg/0.3 µL; filled circles, 16 µg/0.3 µL; open circles, 0.3 µL of vehicle). Arrows mark the time of the injection. Symbols indicate statistically significant differences between a data point and its corresponding preinjection level (* $p < 0.01$; †, $p < 0.001$). Data represent the mean \pm SE for four different animals at each dose. Adapted from Parada et al., 1995.

reward efficacy of EtOH. However, this decrease in reward value was not seen for sucrose following either D_1 or D_2 blockade, suggesting that its reward value may be mediated either by a different neurotransmitter system within the LH or in another substrate altogether. As seen with sucrose, D_1 blockade within the LH also failed to reduce responding for the EtOH solution.

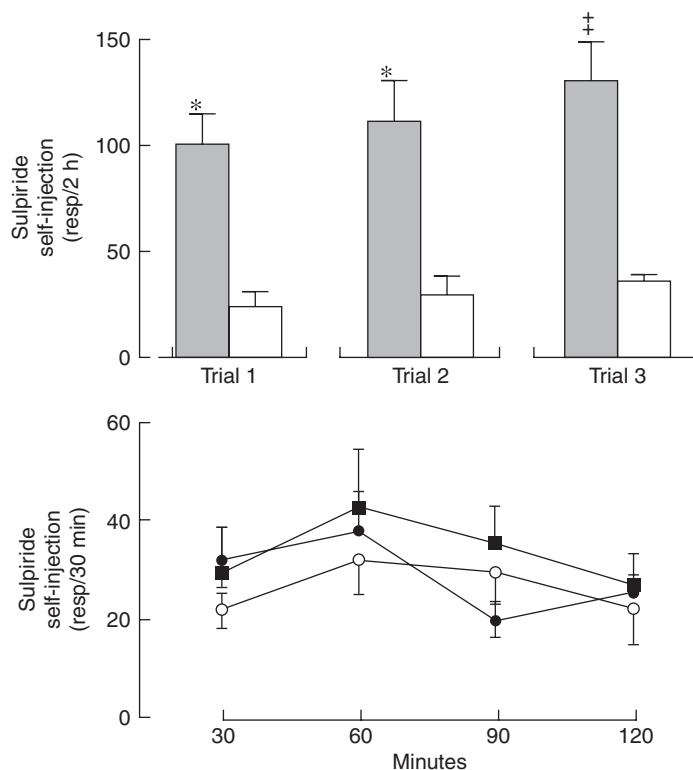


Figure 14.7 Intrahypothalamic self-administration of sulpiride in five rats during sessions (trials) on three different days. Each press of the active lever delivered a 2-s injection of 21.5 ± 1.28 nL of 10 ng/nL sulpiride. Presses on the blank lever were recorded but they did not have any contingent value. Top graph: response frequency on the active lever (filled columns) and the blank lever (open columns) during each session. Symbols indicate significant differences between responses on the active and blank levers (*) $p < 0.01$; ‡, $p < 0.001$). Bottom graph: response frequency on the active lever was consistent in all four 30-min periods during the first (open circles), second (filled circles), and third (filled squares) trials on three different days. Adapted from Parada et al., 1995.

As demonstrated above, the D_1 system produced no change in either EtOH or sucrose responding; however, the absence of effects seen with SCH 23390 may be readily explained. Thus, the LH possesses only a limited number of D_1 receptors [112]. These low numbers may render the D_1 system within the LH ineffective in mediating reinforcement resulting in minimal change in EtOH and sucrose response rates.

While the effects of the D_2 blockade on EtOH self-administration are quite clear, determining the mechanism responsible for this reduction is far more difficult. Nonetheless, plausible explanations are possible. One possible explanation may center around the increased release of accumbal DA previously observed following LH D_2 blockade. As discussed above, D_2 blockade within the LH has been shown to result in increased DA release within the NAcc. This would suggest that such a blockade might in fact increase the reward value of EtOH insofar as increases in

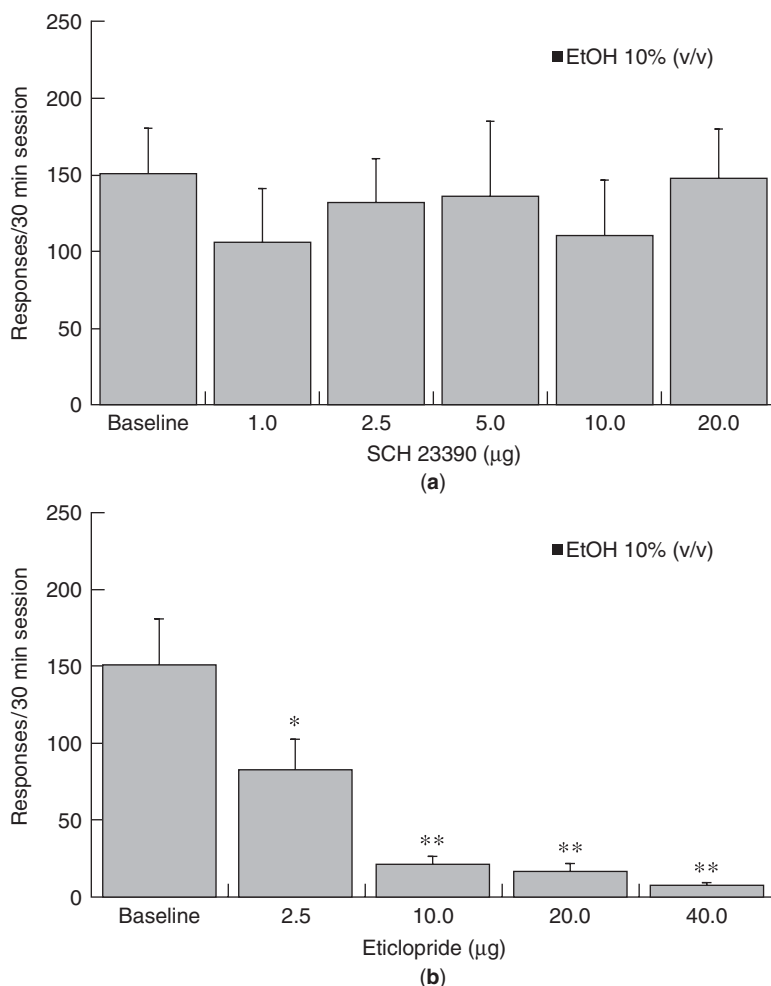


Figure 14.8 (a) Effects of SCH 23390 (1.0 and 20.0 µg) following bilateral injection in the LH on responding maintained by EtOH (10% v/v). Data are shown as mean ± SEM (***) $p < 0.01$ SCH 23390 vs. baseline (noninfusion and saline infusion controls). (b) Effects of eticlopride (2.5–20.0 µg) following bilateral injection in the LH on responding maintained by EtOH (10% v/v). Data shown as mean ± SEM. Adapted from Goergen et al., 2003.

accumbal DA often denote reward efficacy. However, this assumes that the increase in accumbal DA release seen following EtOH self-administration (see [73]) produces an additive effect with the increase in DA release seen with D_2 blockade within the LH [118]. Alternatively, the increased DA release in the NAcc following D_2 blockade within the LH may serve to substitute for the DA release typically seen following EtOH administration. Such a substitution would, in theory, lower the reward value of EtOH resulting in a lowered desire to “work” for the EtOH. This hypothesis, however, fails to explain the effect seen on sucrose consumption. An increase in sucrose responding is expected, because D_2 blockade increases drinking behavior

[114–116]. However, no appreciable change in responding is seen following LH D_2 blockade. It is possible that while the D_2 blockade leads to increased consumptive behaviors, it may not be sufficient to increase the motivation necessary to respond for the sucrose in a self-administration paradigm.

While further research is necessary to identify the mechanisms by which the D_2 antagonist eticlopride reduces EtOH self-administration, it is clear that the D_2 system within the LH strongly regulates the reinforcing properties of EtOH. Indeed, the LH's strong ties to the regulation of the consumptive behaviors of feeding and drinking make it an important site for further EtOH research as alcohol intake can be classified as a drinking behavior. In addition, its clear ability to modulate DA levels within the NAcc may further establish the LH as an important dopaminergic substrate regulating not only EtOH reward but also the rewarding properties of various drugs of abuse.

14.5.4 Summary

The neurocircuitry and mechanisms that mediate the propensity to consume EtOH are complex. The focus in EtOH research thus far has clearly been on the DA system of the mesolimbic pathway, particularly the interaction of the NAcc and the VTA. However, evidence has begun to emerge that may shift the focus from the DA system within the NAcc to other substrates that are innervated by DA neurons arising from the VTA DA cell bodies. The purpose of this section has been to describe novel DA substrates that may be involved in the regulation of EtOH reward. To this end, studies involving three DA-innervated substrates—the BST, VP, and LH—were described in the hopes that future research may involve not only the investigation of the NAcc in EtOH reward but also other dopaminergic brain loci.

Increases in extracellular DA within the NAcc have been observed in a number of studies following the delivery of EtOH through various routes of administration (see [51]). In fact, it has been generally accepted that an increase in extracellular DA following administration is one way to classify a drug as reinforcing. As demonstrated in the studies reviewed above, EtOH administration is capable of inducing elevated extracellular DA levels in the BST and VP. Intraperitoneally administered EtOH at doses that induce euphoric effects has been shown to increase DA release within the BST [48]. A number of studies of DA release in the VP have been conducted by Melendez and colleagues demonstrating that extracellular DA levels increase within the VP following not only i.p. administration but also self-administration in both operant and limited-access paradigms [26, 56, 102]. This group also observed an increase in DA levels following the reverse microdialysis of the D_1 antagonist SCH [26]. While there are no reports of an increase in extracellular DA within the LH, D_2 blockade within the LH is sufficient to produce increases in NAcc DA levels [118]. Although this is not direct evidence that EtOH can influence DA within the LH, it does show that the LH can effectively modulate DA within a substrate shown to regulate EtOH reward.

While the evaluation of extracellular DA is important in establishing a role of a DA system within a substrate in EtOH reinforcement, it does not provide evidence that the substrate can regulate EtOH reinforcement. One method of determining a more direct regulatory role of DA within a brain structure is via use of DA agonists

or antagonists. Using these selective DA agents, it was found that the blockade of the D₁ system within the BST led to robust reductions in EtOH responding with no effect seen with D₂ blockade; therefore, EtOH reinforcement is regulated by D₁ receptor subtypes within the BST [42]. Melendez administered D₁ and D₂ antagonists within the VP via reverse microdialysis and found that the D₁ system had no effect on EtOH reward while blockade of the D₂ system produced increases in intake, suggesting that EtOH reward is mediated by the D₂ receptors within the VP [26]. Different results were seen following microinjection of D₁ and D₂ antagonists into the VP. Using an operant self-administration paradigm, Foster et al. [104] found increases in intake similar to those observed by Melendez, but only at low doses. In contrast, high doses of the D₂ antagonist eticlopride produced robust reductions in EtOH responding. Similar reductions were also observed following the administration of high doses of the D₁ antagonist SCH 23390. These data suggest that EtOH reinforcement may be regulated in a biphasic fashion by D₂ receptors within the VP along with D₁ mediation of reward [104]. The D₂ system also seems to be the regulator of DA reward within the LH. Blockade of the D₂ receptors within the LH led to robust reductions in EtOH responding in a self-administration paradigm [119]. These data demonstrate that while the DA systems in various substrates may mediate EtOH reward efficacy, they do so by the use of differential activation of the D₁ and/or D₂ receptor subtypes.

While the studies discussed in this section detail research on DA's control of EtOH reinforcement in novel substrates, it is important not to discount possible interactions between the DA systems of these brain regions and other neurotransmitter systems. In fact, other studies demonstrate that GABAergic systems can lead to modulation of the effects produced by DA antagonism in both the BST and VP (Eiler et al., unpublished; see also [104]). It is thought that the mechanism of action driving DA regulation of EtOH reinforcement is not simply altering/modulating DA release within key dopaminergic loci but is the result of complex interactions between DA and GABA pathways. Clearly, DA interacts with other neuronal systems such as neuropeptides and glutamate [52]. In short, the elucidation of novel DA substrates regulating EtOH reward provides investigators with new challenges in determining the neurocircuitry that mediates EtOH reinforcement.

In summary, evaluating only DA neuroanatomical substrates and systems within the mesolimbic and extended amygdala pathways may limit our understanding of how the complex interactions within these pathways function to control EtOH-reinforced behaviors. Moreover, it is now clear from the work of both Rassnick et al. [78] and Myers and Quarfordt [120] that removal of the mesolimbic DA system does not alter established EtOH self-administration [121]. As such, other neurotransmitters may be differentially involved in various aspects of the reinforcement process. Since it is likely that EtOH-motivated behaviors are mediated by multiple neurochemical systems, the fundamental neuropharmacological information provided by the study of overlapping neuronal systems is essential to understanding the neural processes that regulate EtOH self-administration. In this review, we describe recent studies evaluating the significance of GABA_A receptors in EtOH-seeking behaviors. These studies have advanced our understanding of the neural mechanisms regulating alcohol drinking. Exploitation of compounds selective for specific GABA_A receptor-containing subunits has now led to novel prototype ligands that may have clinical utility in the treatment of alcohol dependence.

14.6 MOLECULAR BIOLOGY OF THE GABA_A BDZ RECEPTOR

GABA is the most abundant inhibitory neurotransmitter present in the mammalian brain. Although there are, to date, at least three unique classes of GABA receptors, the majority of neurobehavioral research on alcoholism has centered on GABA_A receptors, referred to as the GABA_A benzodiazepine (BDZ) receptor complex. This complex has been characterized as a pentameric structure comprised of at least 16 identifiable subunits (α_{1-6} , β_{1-4} , γ_{1-3} , δ , ρ , and ϵ) in the mammalian CNS [122]. Identifying the various receptor subunits and their function has become increasingly important to gain an understanding of the neurobiological base of alcoholism and treatment target identification. The GABA “system” has been suggested to be the best candidate for a “single” neurotransmitter in regulating the neurobehavioral effects of alcohol [123–125]. Specifically, these effects include alcohol-motivated behaviors [1, 18, 19] as well as the motor-impairing, sedative [28, 124] and anxiolytic [28, 126, 127] properties of alcohol. Here, the neuropharmacological, neuroanatomical, and molecular biological underpinnings of alcohol-motivated behaviors/self-administration are reviewed. The role of the GABA system in other neurobehavioral actions of alcohol has been reviewed elsewhere [124, 128].

14.6.1 Commonalities of Alcohol and Modulators of the GABA_A BDZ Systems

EtOH shares many common behavioral properties with BDZs and barbiturates (e.g., sedation, ataxia, anxiolysis). Similar to BDZs and barbiturates, EtOH potentiates GABA-stimulated Cl[−] flux (for a review see [129]). Hence, it has been postulated that EtOH's action at the level of the GABA-coupled Cl[−] ion channel may underlie many of its behavioral properties [130–132]. The effects of EtOH are thought to be selective for GABA_A receptors since they are antagonized by negative modulators of GABAergic activity such as BDZ inverse agonists, the GABA_A receptor antagonist bicuculline, and the Cl[−] channel blocker picrotoxin [133]. Further, manipulations of central GABAergic activity alters many of the behavioral and physiological effects of EtOH [126, 132, 134, 135], including reinforcement ([78, 136]; also, for a recent review see [19]). Hence, the allosteric modulatory properties of the GABA_A BDZ receptor complex, which allows for neuropharmacological enhancement and reduction of GABAergic function, provides a vehicle to exploit the GABA system to evaluate its significance in regulating alcohol drinking behaviors. Indeed, this neurobehavioral strategy was first demonstrated in the highly cited paper by Suzdak et al. [132] using the partial BDZ inverse agonist RO15-4513. In addition, this neuropharmacological exploitation of the GABA system has been catalyzed by the rapid advances in molecular biology that have resulted in the cloning of key GABA_A receptor subunits [137–140], resulting in knowledge of the heterogeneity and distribution of these receptors [141–143]. For example, substantial research has shown that both the affinities and efficacies of drugs acting at this family of ligand-gated ion channels appear to be defined by subunit composition [137, 144, 145].

The impact of subunit composition on drug action at both recombinant and wild-type GABA_A receptors is best characterized by the chemically diverse class of compounds referred to as the BDZ receptor site ligands [137, 140]. Studies in recombinant GABA_A receptors have shown that the α subunit is the primary determinant of ligand affinity, while the γ subunit affects affinity of a more

circumscribed class of compounds, but can dramatically alter ligand efficacy [139, 144–146]. Hence, knowledge of both the affinity and efficacy of BDZ site ligands has permitted the design/development of pharmacological probes to manipulate the GABA BDZ receptor complex to determine the neuromechanism(s) of action of alcohol drinking behaviors. In addition, the development of these ligands has also provided the opportunity to explore and make inferences about the role GABA systems may play in regulating alcohol drinking behaviors (see below).

14.6.2 GABA_A BDZ Modulation of EtOH Self-Administration: Studies Using Systematic Application of Probes

Substantial evidence now suggests that GABA_A receptor-mediated neurotransmission within the mesocorticolimbic system plays a prominent role in regulating EtOH-motivated behaviors (for a recent review see [19]). Early work demonstrated that GABAmimetics decreased voluntary EtOH intake [147–149], while agents like pentobarbital [150] and diazepam [151] were observed to increase EtOH consumption. In addition, subsequent studies by Amit and his colleagues [152, 153] demonstrated that systemically administered tetrahydroisoxazopyridino (THIP), a GABA agonist, enhances EtOH intake during a 24-h interval. Bretazenil, a partial agonist, has also been observed to increase EtOH intake in AA alcohol-preferring rats [154]. Studies evaluating the role of increased GABA transmission on EtOH-motivated behaviors in the operant chamber, however, have generally resulted in equivocal results on the one hand or no effects on the other. Specifically, Samson and Grant [33], using a concurrent schedule procedure, demonstrated that chlordiazepoxide (CDZ) generally reduced EtOH responding and intake. Rassnick and her colleagues [78] were not able to demonstrate any reliable increases or decreases in EtOH with P and alcohol-non preferring (NP) rats following CDZ (2.5–10 mg/kg) administration. However, using a modified sucrose-fading procedure, Petry [155] demonstrated that low CDZ doses (1–4 mg/kg) significantly and selectively increased EtOH responding, while higher doses (10–20 mg) decreased EtOH responding. In contrast to the equivocal findings evaluating the role of increasing GABA transmission on EtOH self-administration, systemic administration of BDZ inverse agonists [e.g., RO15-4513, FG 7142, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM)] have been consistently reported to reduce EtOH consumption under a variety of self-administration paradigms (for reviews see [156, 157]). For example, RO15-4513 has generally been reported to dose dependently and selectively suppress EtOH intake [7, 156, 158] and responding [78, 159, 160].

Because of the demonstrated selectivity of RO15-4513 in reducing alcohol intake, subsequent work focused on RO19-4603, a related BDZ inverse agonist with a unique pharmacological profile [20, 161]. Unlike RO15-4513, RO19-4603 is an imidazothienodiazepine derivative of RO15-3505 (samazenil), with exceptionally high binding affinity ($K_i = 0.2$ nM) [162]. Figures 14.9a and b show data for P rats ($N = 10$) which initiated EtOH-reinforced responding using a modification of the sucrose-fading procedure under a concurrent FR4 operant schedule for days 1 and 2. RO19-4603 (0.0045–0.3 mg/kg, i.p.) was given 5 min prior to day 1 only. RO19-4603 reduced response-contingent EtOH intake by as much as 97% of controls on day 1, and responding continued to be reduced 24 h post-drug administration by as much as 85% of controls on day 2. CGS 8216, a specific BDZ antagonist, significantly attenuated the RO19-4603 reduction on day 1 and completely reversed the effects on

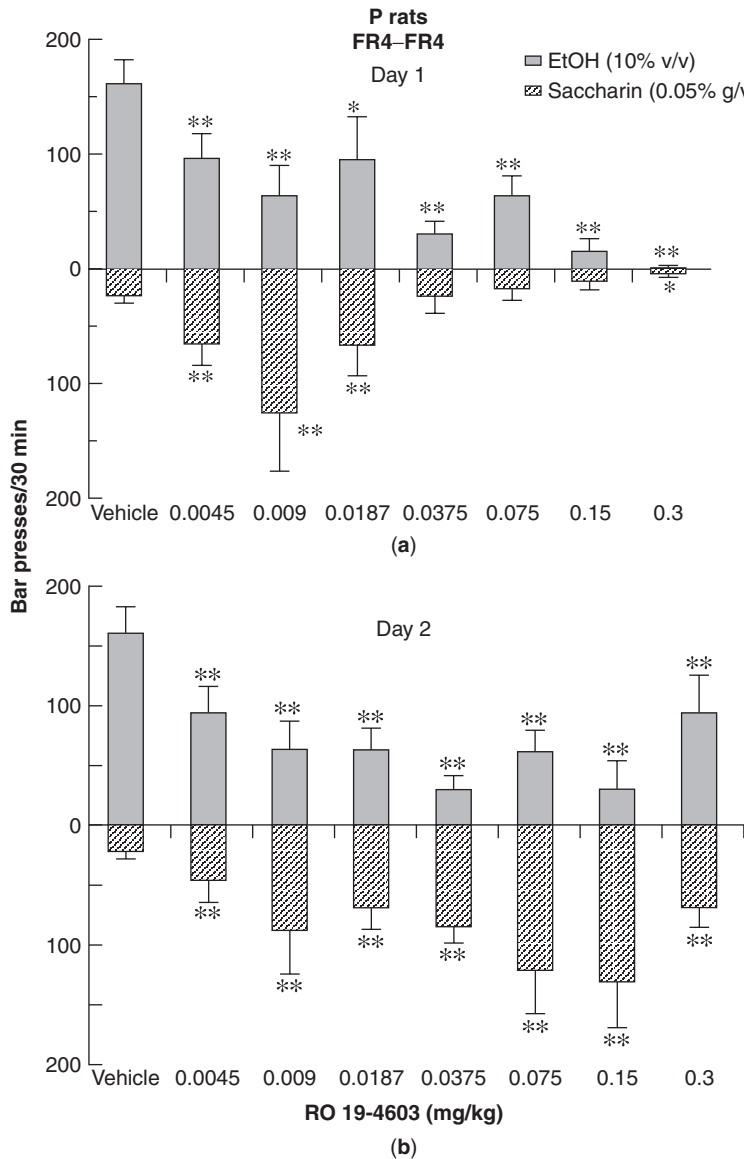


Figure 14.9 Dose-response and time course of i.p. administration of RO19-4603 (0.0045, 0.009, 0.0187, 0.0375, 0.075, 0.15, 0.3 mg/kg) and vehicle given acutely on day 1 (a) and 24 h post-drug administration day 2 (b) on EtOH-reinforced (10% v/v) responding and saccharin-reinforced (0.05% g/v) responding. The RO19-4603 injections were given 5 min prior to test session on day 1 only. RO19-4603 suppressed EtOH-reinforced responding, while the 0.0045-, 0.009-, and 0.0187-mg/kg doses elevated saccharin-reinforced responding on day 1. Day 2 showed suppression of EtOH-reinforced responding and parallel increases of saccharin-reinforced responding for each of the seven doses of RO19-4603. (*) $p < 0.05$ and (**) $p < 0.01$ vs. control vehicle values by ANOVA and post hoc Newman-Keuls test at a corresponding day. Adapted from June et al., 1998.

day 2. Thus, the effects of RO19-4603 may be mediated at the BDZ binding site of the GABA_A BDZ receptor complex. The degree of selectivity of RO19-4603 on EtOH responding, however, could not adequately be determined from this study, primarily because EtOH and saccharin responding were not equated at basal levels; however, analyses of the cumulative records revealed that RO19-4603 produced a dose-dependent decrease in the slope of the cumulative record for EtOH responding while concomitantly producing a dose-dependent increase in the slope for saccharin responding (see Fig. 14.10). Thus, the actions of RO19-4603 “appear” to be mediated via recognition sites at GABA_A BDZ receptors which regulate EtOH reinforcement, and not mechanisms regulating general ingestive behaviors.

In the evaluation of BDZ ligands for their effectiveness to attenuate EtOH responding, June and colleagues have determined if qualitative (i.e., imidazobenzodiazepine-selective) and quantitative (affinity for diazepam insensitive (DI) sites; see [144, 162a]) differences exist in a compound's ability to decrease EtOH responding (see [28]). RU 34000 is a novel imidazopyrimidine inverse agonist with relatively low affinity at BDZ receptors ($K_i \sim 0.98 \mu\text{M}$) [163] and little affinity at DI sites [164]. Pharmacokinetic analyses, however, have shown that this low affinity is compensated for by the high drug levels of RU 34000 in the brain ($10 \mu\text{g/mL}$) [165]. RU 34000 was an effective antagonist of EtOH responding via the i.p. route. Effects on saccharin responding were seen only with the highest i.p. dose (5 mg/kg). Flumazenil (6 mg/kg , i.p.) completely attenuated the RU 34000 reduction of EtOH responding (data not shown), suggesting that the actions of RU 34000 may be mediated at central BDZ sites. The data with RU 34000 allow several interpretations: First, in addition to the affinity of BDZ ligands at active CNS sites, their bioavailability is an important factor in their capacity to function as EtOH antagonists; second, the capacity of BDZ ligands to function as effective EtOH antagonists is not specific to imidazobenzodiazepines; and third, it is likely that the DI GABA_A receptors (i.e., those receptors either an α_4 or α_6 subunit) may not be a critical factor regulating EtOH-maintained responding.

Work with several BDZ antagonists has also revealed some interesting findings using operant methodology [166]. Figure 14.11 shows that unlike the prototypic BDZ antagonist RO15-1788 (flumazenil), the pyrazoloquinoline CGS 8216 ($1\text{--}20 \text{ mg}$) and the β -carboline antagonist ZK 93426 ($5\text{--}50 \text{ mg/kg}$) produce marked suppressant effects on EtOH responding [166, 167]. Further, 24 h post-drug administration, nonsignificant effects were seen with the lower doses and significant effects with some of the higher doses of these agents [see 161, 168]. It should be noted that, like flumazenil, both ZK 93426 and CGS 8216 are well tolerated in human subjects [169, 170], and it is possible that these agents may have some capacity to reduce EtOH drinking in humans. The exact mechanism by which BDZ antagonists reduce EtOH drinking is not clear. Mild agonist effects for ZK 93426 have also been reported in vivo in rats [171] and humans [172]. In rats, CGS 8216 produces weak to moderate anxiogenic effects, resembling a weak partial inverse agonist [173]. Moderate to strong agonist effects, however, have been consistently reported in vitro in the *Xenopus* oocyte assay for ZK 93426 [18, 37] (see below). Thus, one possible interpretation of the antagonism of EtOH drinking by ZK 93426 and CGS 8216 is that a “BDZ agonist-like ligand” substitutes for the reinforcing actions of EtOH [78], as has been reported with DA agonists in blocking the reinforcing properties of EtOH (cf. [17]). Nevertheless, because of the potential clinical applications of BDZ antagonists,

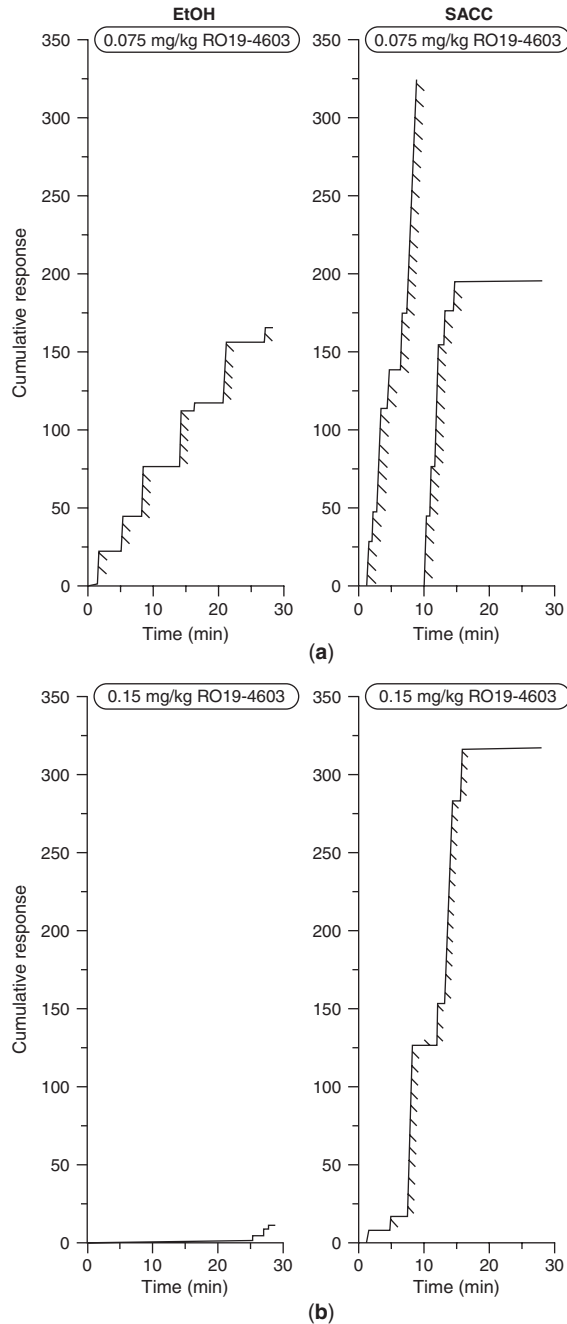


Figure 14.10 Sample cumulative dose-response records for EtOH (10% v/v) and saccharin (0.05% g/v) responding in two additional P rats during a concurrent schedule (FR4–FR4) following 0.075 mg/kg of RO19-4603 (a) (upper panel) or 0.15 mg/kg of RO19-4603 (b) (lower panel). Cross-hatch equals delivery of reinforcer, while the slope of lines indicate response per rate per minute. Adapted from June et al., 1998.

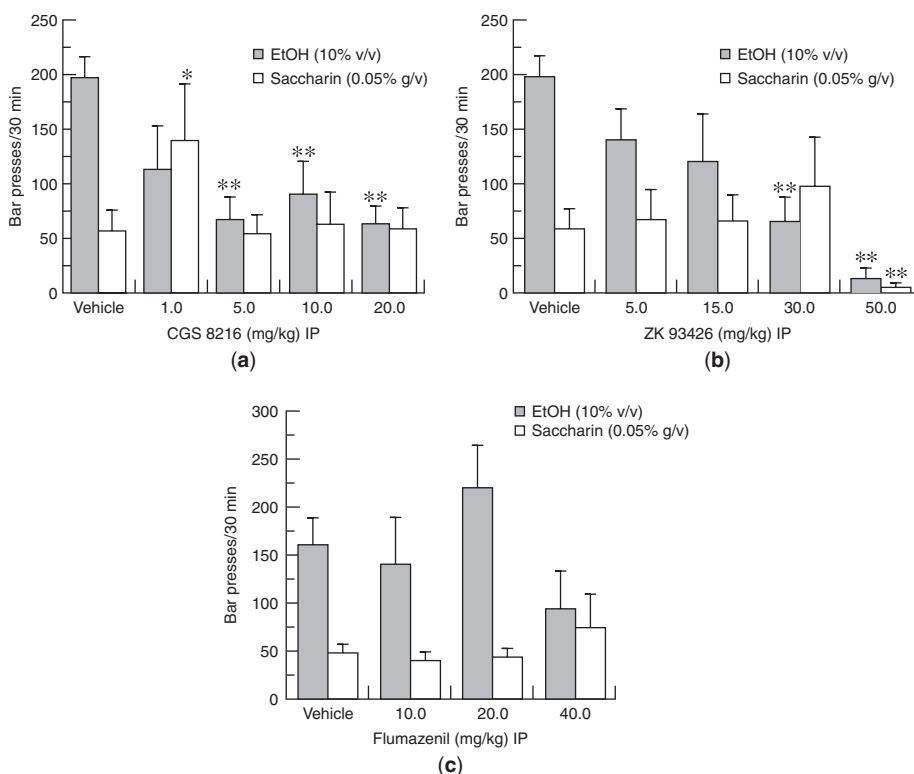


Figure 14.11 (a) Dose–response of i.p. administration of flumazenil in doses of 0 (Tween-80), 10, 20, and 40 mg/kg on responding maintained by EtOH (10% v/v) and saccharin (0.05% g/v) (FR4-FR4). The flumazenil injections were given 20 min before the test sessions ($n = 10$). (b) Dose–response and time course of i.p. administration of CGS 8216 (1, 5, 10, and 20 mg/kg) and vehicle on responding maintained by EtOH (10% v/v) and saccharin (0.05% g/v) (FR4-FR4). The CGS 8216 injections were administered 20 min before the test session. (c) Dose–response and time course of i.p. administration of ZK 93426 (5, 15, 30, and 50 mg/kg) and vehicle on responding maintained by EtOH (10% v/v) and saccharin (0.05% g/v) (FR4-FR4). The ZK 93426 injections were administered 20 min before the test session. (*) $p < 0.05$ and (**) $p < 0.01$ vs. control vehicle values by ANOVA and post hoc Newman–Keuls testing at a corresponding day ($n = 10$). Adapted from June et al., 1998.

additional studies are warranted to further understand the mechanism by which they decrease EtOH reinforcement. Another hypothesis regarding the capacity of BDZ antagonists to attenuate alcohol intake is that because they “generally” do not result in a functional increase or decrease in GABAergic activity, it is possible that their effects on EtOH responding are likely due to their binding at various GABA_A-containing receptor subunits [19, 166].

14.6.2.1 Significance of Systemic Studies. Systemic administration of RO19-4603 produced clear dose-related reductions on EtOH-maintained responding. Compared with other inverse agonists [e.g., RU 34000, Samazenil, ethyl beta-carboline-3 carboxylate (β CCE), RO15-4513], RO19-4603 was considerably more potent, selective, and long lasting in reducing EtOH-maintained responding. Cumulative dose-response profiles

revealed that RO19-4603's actions on EtOH responding appear to be mediated via recognition sites on the GABA_A/BDZ receptor complex which regulate EtOH reinforcement, and not via mechanisms regulating ingestive behaviors in general (see Fig. 14.9). Moreover, the prolonged effects of RO19-4603 on EtOH self-administration was also observed in outbred rats for at least 48 h after a single administration [174], suggesting that these effects are not specific to the P rat line. Attenuation of the RO19-4603-induced suppression on days 1 and 2 by a high-affinity, competitive BDZ antagonist suggests that this effect on EtOH-maintained responding is mediated at the BDZ component of the GABA_A/BDZ receptor complex. Thus, RO19-4603 may be the ideal pharmacological tool to microinject into novel CNS sites to evaluate the role of GABAergic neurotransmission in EtOH reinforcement.

The demonstration that selected BDZ antagonists can blunt the reinforcing properties of EtOH suggests a direct role for the BDZ binding site on the GABA_A BDZ receptor complex in EtOH-seeking behavior. The precise mechanisms by which BDZ antagonists reduce EtOH drinking, however, are not clear, primarily because these studies employed systemic injections. Using the microinjection technique, however, it may be possible to more precisely delineate the neurobiological mechanism(s) in regulating EtOH reinforcement, particularly in a well-characterized model of EtOH-seeking behavior (e.g., P and HAD rats). Finally, because "neutral competitive" BDZ ligands produce few untoward effects in animals and humans ([169, 170] see [166]), they represent excellent pharmacological tools to explore the neurobiology of alcohol reinforcement. A review of the literature on BDZ competitive ligands (see [169, 170]) also shows that neither ZK 93426 nor CGS 8216 produces untoward effects in humans, further supporting the utility of its use in alcohol drinking studies.

14.6.3 Site-Specific Microinjection Studies: Manipulation of GABA_A BDZ Receptor Complex in Modifying EtOH Self-Administration

Little is known about the neurochemical and neuroanatomical substrates via which manipulation of the GABA_A BDZ receptor complex can modify EtOH self-administration within the mesolimbic/mesoaccumbens system. The initial paper to appear in the published literature [55] examined the role of GABA_A receptors in the extended amygdala on EtOH-reinforced responding in outbred rats. As noted previously, the extended amygdala comprises the central nucleus of the amygdala, the bed nucleus of the stria terminalis and the shell of the NAcc, and the subnucleus SI [175]. Animals were trained to initiate EtOH intake using the saccharin-fading procedure. The effects of bilateral microinjections of the competitive GABA_A receptor antagonist SR 95531 were evaluated in three of these brain loci. SR 95531 injected in the bed nucleus of the stria terminalis reduced responding for EtOH with the 8- and 16-ng doses, while only a 16-ng dose produced a significant effect in the shell of the NAcc. However, injections into the bed nucleus of the stria terminalis and the NAcc suppressed both EtOH and water responding at the highest doses during the initial part of the drinking session. Intra-amygdaloid injections did not disrupt the initiation of responding. These findings suggested that the GABA_A receptors in the extended amygdala may be involved in the mediation of some aspects of EtOH reinforcement. Hyttiä and Koob [55] contend the differential sensitivity of the central amygdala, bed nucleus, and shell of the NAcc may result from differences in density

of GABAergic neurons. Indeed, the NAcc contains fewer GABAergic neurons than the bed nucleus of the stria terminalis or the central amygdala [88]. It is important to note, however, that reinforcer specificity could not accurately be determined in that study because rats responded for a (nonpalatable) water solution and responding for the water solution was so low as to confound ready interpretation.

GABAergic manipulations within the mesocorticolimbic DA systems have been investigated primarily with the GABA_A agonist muscimol and the GABA_A antagonist bicuculline. Hodge et al. [176] hypothesized that since local infusions of muscimol into the VTA had been previously shown to increase DA in the NAcc [177], it was possible that such interactions could modulate (directly or indirectly) EtOH-reinforced responding. Hodge et al. [176] evaluated the effects of bilateral microinjections of muscimol (1–30 ng) and bicuculline (1–10 ng) in the NAcc of outbred rats after they were trained under the sucrose-fading procedure. The 10- and 30-ng doses of muscimol significantly decreased EtOH responding. Similarly, the 3- and 10-ng doses of bicuculline also reduced responding. When a dose of bicuculline (1 ng, a dose that had no effect on EtOH given alone) was coadministered with muscimol, it attenuated the muscimol-induced decrease in EtOH responding. Together, these findings suggested that the effects seen with muscimol were mediated by activity at GABA_A receptors and that GABAergic transmission in the NAcc is involved in EtOH reinforcement, possibly via inhibition of DA function [176]. In a subsequent study, these same researchers [178], employing similar methods, evaluated in the VTA the effects of bilateral infusions of higher doses of muscimol (10, 30, and 100 ng) on EtOH (10%, v/v) and sucrose (75%, w/v) responding in separate groups of rats. The results showed that muscimol (10 ng) increased the number of sucrose responses but had no effect on the total number of EtOH responses. However, the 30-ng dose shifted the response pattern of both groups from high initial rates with early termination to slow initial rates with delayed termination, suggesting nonspecific effects. Nevertheless, taken together, these data suggest that muscimol differentially alters EtOH and sucrose responding via GABAergic mechanisms within the VTA, in that EtOH reinforcement is less sensitive to GABAergic manipulation compared with sucrose responding. These investigators employed a very high sucrose concentration in the VTA study; hence, the efficacy of the alcohol and sucrose is very difficult to equate, particularly in outbred rats (see [4]).

To further investigate the role of the GABA system in the mesoaccumbens system, June and colleagues [161, 179] conducted several studies with RO19-4603. The main objectives of these studies were to evaluate, first, its prolonged time course of alcohol reduction in the operant chamber and, second, the substrate by which it exerted its effects. Figure 14.12 shows data for P rats trained to bar press for EtOH under the saccharin-fading procedure following unilateral intra-accumbens infusions of RO19-4603 (2–100 ng). Similar to the systemic study (see above), infusions were performed *only* on day 1. Microinjections of RO19-4603 (20 and 100 ng) into the NAcc suppressed EtOH responding on day 1 by as much as 53% of control, and responding continued to be suppressed on day 2, with the 100-ng dose reducing responding to 73% of control. Thus, similar to systemic injection, prolonged effects were observed with RO19-4603. Cason et al. [180] examined outbred Wistar rats following unilateral injections of *tert*-butyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5*a*] [1,4]-benzodiazepine-3 carboxylate (TG) on day 1 and 24 h post-drug administration. Note that RO19-4603 is also a *t*-butyl ligand; however, TG

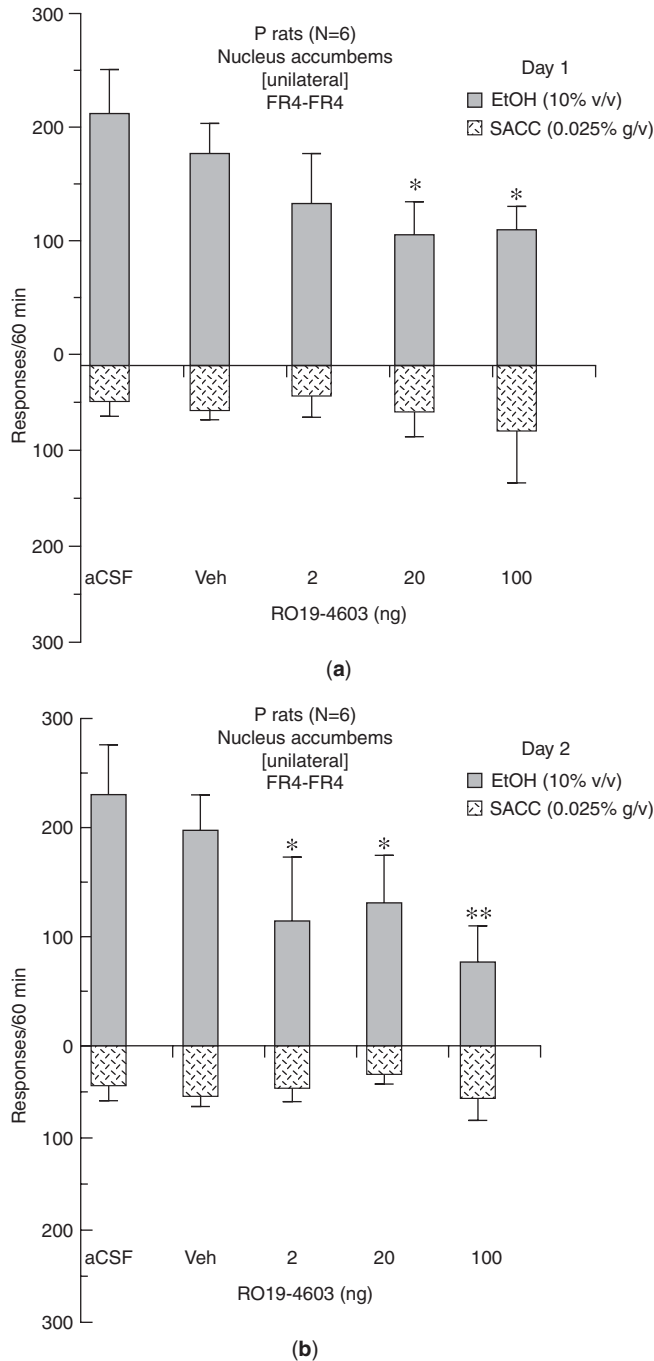


Figure 14.12 Time course of intra-accumbens infusion of RO19-4603 [aCSF (0.0), 2, 20, 100 ng] given acutely on day 1 (a) and 24 h post-drug administration day 2 (b) on EtOH (10% v/v) and saccharin-reinforced (0.05% g/v) responding. (*) $p < 0.05$ and (**) $p < 0.01$ vs. aCSF and vehicle control condition values by ANOVA and post hoc Newman-Keuls test at a corresponding day. Adapted from June et al., 1998.

differs from RO19-4603 in that it is a *t*-butyl acetylene. TG was synthesized by Cook and colleagues for comparison with RO19-403 in our EtOH-maintained responding studies. Following unilateral infusions of TG into the accumbens, the lower dose of TG (200 ng) selectively reduced responding on day 1, but the higher dose (500 ng) nonselectively reduced both EtOH and saccharin responding. On day 2 (24 h postdrug), TG continued to selectively reduce EtOH responding without altering saccharin-maintained responding. The 24-h post-drug administration results with TG replicate the long time course effects on EtOH-maintained responding seen with RO19-4603 in P rats. It should be noted that both EtOH and saccharin response rates were similar at basal levels in the Wistar rats. However, unlike the RO19-4603 study, rate dependency effects cannot adequately explain the efficacy of TG as an EtOH antagonist [180]. These data suggest [168] that the pharmacokinetics of such ligands may be important in their long duration of action since typical ester-derived agents (e.g., RO15-4513, RY 008) are short-lived *in vivo* since they are readily deactivated by esterase enzymes. This is not the case with the *t*-butyl analogs since they cannot readily be deactivated by such enzymes. Consequently, *t*-butyl agents may exhibit a more prolonged duration of action *in vivo* [181]. These data are interesting insofar as modification of such agents to eliminate their negative efficacy could result in prototypes which may reduce alcohol consumption over a long time course.

P rats were administered bilateral microinjections into the VTA using the inverse agonist RU 34000. The purpose of this experiment was to determine if a non-imidazobenzodiazepine inverse agonist could also attenuate EtOH responding via the GABAergic DA reward circuitry. Clear, dose-dependent suppressant effects were observed on EtOH responding following RU 34000 (50–200 ng) in the absence of any effects on saccharin responding. Similar to the systemic studies (see [168]), no effects of RU 34000 were observed on day 2. These data suggest that RU 34000 may be a useful tool to further investigate the effects of the GABA_A BDZ systems in mediating EtOH reward in the VTA and that an imidazobenzodiazepine structure may not be critical to observe reduction on alcohol intake within the mesolimbic circuitry.

These findings allow for several interpretations: (1) GABAergic transmission through GABA_A BDZ receptors contributes to the mediation of EtOH reinforcement in the mesoaccumbens limbic circuitry; (2) in rodents, high voluntary EtOH consumption does not appear to be linked to the reinforcing properties of EtOH in a manner that would permit GABAmimetics or BDZ agonists to substitute for EtOH; and (3) the specific neuroanatomical circuits participating in GABAergic modulation of EtOH self-administration cannot be determined from systemic studies, primarily because of the widespread distribution of GABA_A receptors located in the CNS. Hence, additional microinjection studies are needed to further identify central GABAergic mediation of EtOH reward sites.

14.6.4 Studies Supporting Hypothesis That GABA–DA Interactions Regulate Alcohol-Motivated Behaviors: GABAergic Modulation of DA Function

The VTA receives GABAergic inputs from the medial prefrontal cortex (MPC), VP, and NAcc [182]. In addition, it is well established that substantial GABAergic interneurons exist within the VTA [64]. These GABAergic projections appear to modulate activity of DAergic neurons with cell bodies localized within the VTA. Ikemoto et al. [183] demonstrated that microinjections of picrotoxin (a nonselective

GABA antagonist) into the anterior VTA markedly increased extracellular DA in the NAcc. In anesthetized rats, Yim and Mongenson [184] reported that GABA inhibited the discharge rate of DAergic neurons in the VTA, while application of picrotoxin increased the discharge rate of DAergic neurons. Hence, converging evidence suggests that GABA_A receptors in the VTA mediate tonic inhibition over DAergic neurons. In addition, VTA DAergic neurons that project to the NAcc appear to be activated by blockade of GABA_A receptors. Thus, since the projection from the VTA to the NAcc (mesoaccumbens DA system) has been implicated in reward-related processes [185, 186], one possible mechanism that underlies the blockade of EtOH-reinforcing effects by BDZ inverse agonists and specific GABA_A receptor antagonists may be activation of the mesoaccumbens DA system and, thereby, enhanced reinforcing effects.

While a vast and mature literature exists on the interactions between brain GABA and DA systems (see [187] for a review), the modulation of this interaction by BDZ ligands and, more specifically, inverse agonists is not as extensive (for a review see [188]). In general, these data suggest that inverse agonists can mimic the enhancing effects of stress on DA transmission in the prefrontal cortex. Specifically, systemically administered β -carbolines [e.g., beta-carboline-3-carboxylate methyl ester (β CCM), β CCE, FG 7142] have been shown to selectively increase DOPAC, or DOPAC–DA ratios [189, 190]. This increase in DA transmission has been more directly measured as an increase in cortical DA efflux using microdialysis techniques [191]. It is also worth noting that the EtOH antagonist RO15-4513 also increases prefrontal DOPAC [190]. The precise neuronal circuits underlying the BDZ modulation of cortical DA transmission, however, remains unclear because the vast majority of the studies have employed systemic drug injections (for a review see [188]). The effects of inverse agonists on subcortical DA neurotransmission have also been investigated. McCullough and Salamone [192] demonstrated that the inverse agonists FG 7142 (10–30 mg/kg) or β CCE (1.25–2.5 mg/kg) led to dose- and time-dependent increases in DA efflux. Coco et al. [193] examined BDZ ligand modulation in stress-induced DA transmission in the mesoamygdaloid system. Conditioned footshock increased homovanillic acid content in several regions of the amygdaloid and septal nuclei in the absence of any effects in cortical areas. Moreover, these effects were antagonized by diazepam. Collectively, these studies demonstrate the capacity of GABA antagonists and BDZ inverse agonists to modulate cortical and subcortical DA systems and provide support for proposing to use these agents as neuropharmacological tools to investigate GABAergic modulation of DA function.

14.6.5 Conceptual Framework for Hypothesis Generation and Interpretation of GABA–DA Interaction in Alcohol Drinking Behavior

It has become increasingly clear that alcohol-motivated behaviors in selected and outbred rat lines are regulated by multiple neurotransmitter systems [1, 9]. As stated previously in this review, a major task for alcohol self-administration investigators will be to determine the extent to which these neuronal systems singly and collectively regulate alcohol-seeking behaviors. We propose that GABAergic neurons may regulate alcohol's euphoric properties via the involvement of GABA within (in the VP) mesolimbic DA or opioid systems [1, 194]. The topography of the VP [64, 195] places it in a unique position to serve as a pivotal regulator of dopaminergic,

opioidergic, and GABAergic inputs that could control EtOH-motivated behaviors. However, the close proximity and coexistence of GABA and DA within this reward circuitry [177, 196, 197] makes DA a more likely candidate to interact with the GABA system. Previously, Legault et al. [198] have established a DA link between the ventral hippocampus (i.e., subiculum) NAcc and VTA, with DA activation being dependent on initial stimulation of the hippocampus and integrity of the NMDA receptor. We will argue (and present indirect evidence from the literature) that a negative GABA modulator, in particular one selective for the hippocampus, may augment DA to regulate reward-related behavior. In addition, VP GABAergic neurons have been shown to mediate the major DA output neurons of the VP [199]. Thus, we hypothesize in this review that both the VP and hippocampus, because of their high preponderance of GABA α_1 and α_5 receptor subunits, respectively, represent unique substrates to begin systematically testing a GABA–DA interaction hypothesis of alcohol-motivated behaviors. Finally, we will argue that an attempt to understand the GABA–DA interaction in the VP alone would be a gross oversimplification of how this complex interactional pathway functions to mediate alcohol-rewarded behavior.

14.6.6 Novel CNS GABAergic Substrates Regulating Alcohol-Motivated Behaviors

Despite the growing body of evidence for the GABA system in regulating EtOH reinforcement, much remains unknown about the role of specific GABA_A receptor subtypes. This primarily reflects (1) the paucity of high-affinity and selective ligands capable of discriminating among GABA_A receptors and (2) the heterogeneity of various subunits within the known alcohol reward circuitry [141, 143]. Figure 14.13 depicts this heterogeneity of the α subunits (the principal determinant of BDZ ligand affinity) within the alcohol reward loci using in situ hybridization and immunocytochemistry findings [141–143]. However, despite the remarkable heterogeneity within and among the brain reward loci, several substrates exhibit a preponderance of a single α subunit. These structures can be exploited for investigation of alcohol's rewarding effects. The probability of accurately delineating such structures in alcohol reward is markedly enhanced when a selective (albeit low- to moderate-selectivity) GABA_A receptor subtype ligand is employed.

14.6.7 Ligands with Preferential Selectivities for GABA_A Receptors Containing α_1 Subunit (GABA_{A1} Receptors)

β -Carboline-3-carboxylate-*t*-butyl ester (β CCt) and 3-propoxy- β -carboline hydrochloride (3PBC) were initially synthesized as “neutral” β -carboline antagonists [122, 200–202] with selectivity at the α_1 -containing GABA_A receptors (also known to as the BDZ1 receptor). Behavioral studies in several species (e.g., rats, mice, primates) show that both ligands can function as BDZ antagonists, exhibiting competitive binding site interaction with BDZ agonists over a broad range of doses [201–208]. Other studies have shown that both ligands produce anxiolytic effects in rodents [203], while β CCt potentiates the anticonflict response induced by α_1 subunit ligands in primates [205]. Thus, both β CCt and 3PBC are capable of displaying an agonist or antagonist profile depending on the behavioral task, species, and dose employed.

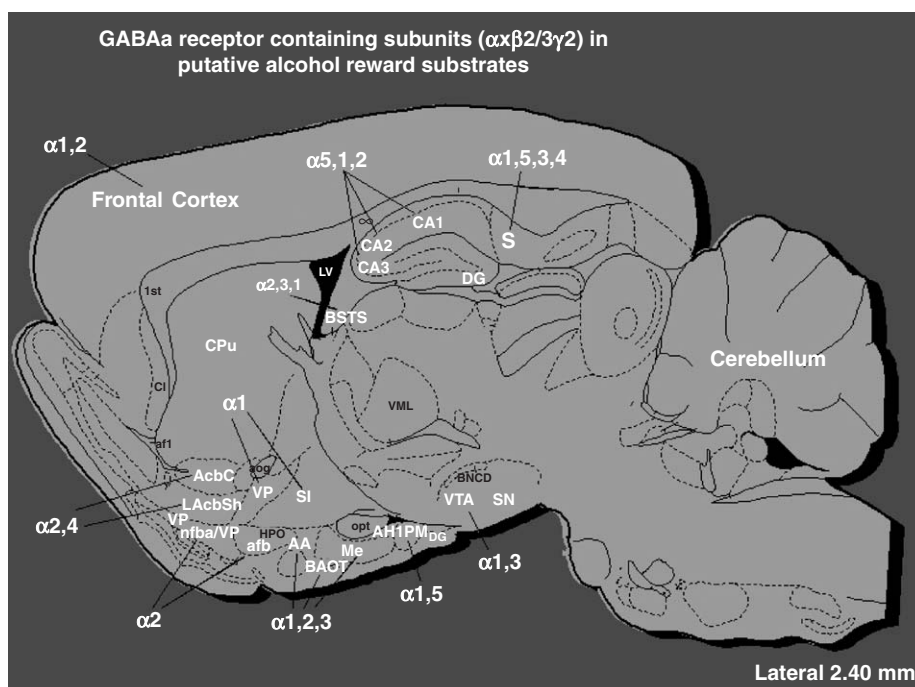


Figure 14.13 Distribution of GABA_A receptor containing subunits (e.g., $\alpha\beta 2/3\gamma 2$) in putative alcohol reward substrates based on in vitro immunocytochemistry [141, 142, 146], in situ hybridization [143, 146, 213, 214], and in vivo neurobehavioral [18, 19, 30, 37, 123, 125] studies. (See color insert.)

Hence, their behavioral pharmacological profile can best be described as that of “a partial agonist–antagonist” [19, 37].

Until recently, zolpidem and CL 218, 872 were the most selective ligands for the α_1 subunit (see [200]). Studies of recombinant receptors show βCCt exhibits greater than 10-fold selectivity for the α_1 over the α_2 and α_3 subunits and more than 110-fold selectivity for the α_1 over the α_5 subunit [19, 200] (see Table 14.1). Zolpidem has only a 5- and 15-fold greater selectivity at the α_1 subunit compared with α_2 and α_3 subunits, respectively. Neither βCCt nor zolpidem have significant affinities at the α_5 and α_6 subunits. 3PBC also displays a moderate level of selectivity for the α_1 subunit, exhibiting 9.8-, 13-, and 111-fold selectivity relative to the α_2 , α_3 , and α_5 subunits, respectively [37] (Table 14.1). Hence, βCCt and 3PBC exhibit the greatest binding selectivity of the currently available α_1 -subunit ligands reported to date [201, 209, 210].

14.6.8 Efficacies of βCCt and 3PBC in Modulating GABA Responses in Recombinant GABA_{A1,2,3,5} Receptors

Unlike the pharmacological profile of many other neuronal system ligands, the selectivity of GABA BDZ site ligands can also be described in relation to pharmacological efficacy. This efficacy is based on the capacity of a ligand to modulate GABAergic function [137]. As discussed below, there is some debate

TABLE 14.1 Affinity of α_1 (Top) and α_5 (Bottom) Selective Ligands

Compound	α_1	α_2	α_3	α_5	α_6 (DI), nM
β CCT	0.7	15	18.9	111	> 5,000
3PBC	5.3	52.3	68.8	591	> 1,000
3EBC	6.4	25.1	28.2	826	> 1,000
Zolpidem	27	156	383	10,000	> 10,000
CL 218, 872	57	1960	1160	561	> 10,000
Diazepam	14	20	15	11	> 3,000
ZK 93426	11	31	24	3	1,600
L-838,417	0.79	0.67	0.67	267	2.25
RO15-4513	3.3	2.6	2.5	0.26	3.8
RY023	197	143	255	2.61	58.6
RY024	27	26.3	18.7	0.4	5.1
RO15-1788	0.8	0.9	1.05	0.6	148

Note: In vitro **recombinant binding assay**. The affinity of compounds at GABA_A/BDZ receptor, subtypes was measured by competition for [³H]RO15-1788 (83 Ci/mmol; NEN) binding to Ltk cells expressing human receptors of composition $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$, and $\alpha_6\beta_3\gamma_2$. Cells were removed from culture by scraping into phosphate-buffered saline, centrifuged at 3000 g and resuspended in 10 mL of phosphate buffer (10 nM KH₂PO₄, 100 nM KCl, pH 7.4 at 4°C) for each tray (25 cm²) of cells. Radioligand binding assays were carried out in a volume of 500 μ L which contained 100 μ L of cells, [³H]RO15-1788 at a concentration of 1–2 nM and test compound in the range of 10^{−9}–10^{−5} M. Nonspecific binding was defined by 10^{−5} M diazepam and typically represented less than 5% of the total binding. *K*₁ values were calculated using the least-squares iterative fitting routine of RS/1 analysis software (BBN Research Systems, Cambridge, MA) and are either mean \pm SEM (standard error of the mean) or the means of two determinations which differed by less than 10%. Recombinant receptors expressed with either a β_3 or β_2 subunit have been shown to exhibit the same benzodiazepine receptor ligand affinities [37, 146, 246, 247].

in the literature as to whether a ligand's affinity or efficacy selectivity is the more salient factor in determining a ligand's capacity to function as an alcohol antagonist [4, 18, 123]. Hence, knowing the efficacy of putative anti-alcohol reward ligands across GABA_A receptors is indeed critical to knowledge of their neuromechanism of action. This is particularly the case with BDZs, insofar as ligands may have a different efficacies depending on their activity at a particular subunit. Hence, the efficacy of β CCT and 3PBC's has been investigated across all "diazepam-sensitive" receptors (i.e., receptors bearing α_1 , α_2 , α_3 , or α_5 subunits) and the α_4 subunit in the *Xenopus* oocyte assay [4, 37]. For comparison, the activities of the prototypical antagonists ZK 93426 and flumazenil were also evaluated. Figure 14.14 shows that β CCT exhibited either a neutral or low-efficacy agonist response at GABA α_1 (96 \pm 7%), α_2 (99 \pm 10%), α_3 (108 \pm 6%), and α_4 (107 \pm 5%) subunits compared to a normalized GABA response of 100% in its absence. However, a low-efficacy partial inverse agonist response was observed at the α_5 subunit (88 \pm 7% of the GABA response). Flumazenil exhibited an efficacy profile that was qualitatively similar to β CCT at the α_1 (99 \pm 5%), α_3 (118 \pm 7%), and α_5 (96 \pm 6%) subunits. At the α_2 subunit, flumazenil produced a low-efficacy agonist response (115 \pm 4%) while β CCT was GABA neutral (98 \pm 10%). Flumazenil also produced a qualitatively similar response to β CCT at the α_4 subunit, albeit the magnitude of GABA potentiation by

flumazenil far exceeded that of β CCt ($132 \pm 6\%$ vs. $108 \pm 6\%$, respectively). In contrast, ZK 93426 produced a clear agonist profile, potentiating GABAergic activity (see below).

3PBC acted as a modest positive modulator at α_1 -, α_2 -, α_3 -, and α_4 -containing receptors ($113 \pm 4\%$, $116 \pm 7\%$, $119 \pm 6\%$, $129 \pm 3\%$ of GABA response, respectively). At the α_1 through α_5 subunits, flumazenil exhibited an efficacy profile statistically similar to 3PBC ($p > 0.05$). At the α_1 through α_4 subunits, ZK 93426 exhibited a full agonist profile ($146 \pm 11\%$, $140 \pm 13\%$, $147 \pm 10\%$, $137 \pm 8\%$, respectively). These effects were statistically greater than 3PBC and flumazenil at the α_1 through α_3 subunits ($p < 0.05$). By comparison [211, 212], flunitrazepam, the full agonist, markedly enhanced GABAergic activity ($152 \pm 8\%$ to $164 \pm 3\%$) across the α receptor subunits (data not shown). At the α_5 subunit, each of the three antagonists exhibited a very weak negative profile which was indistinguishable from each other ($p > 0.05$).

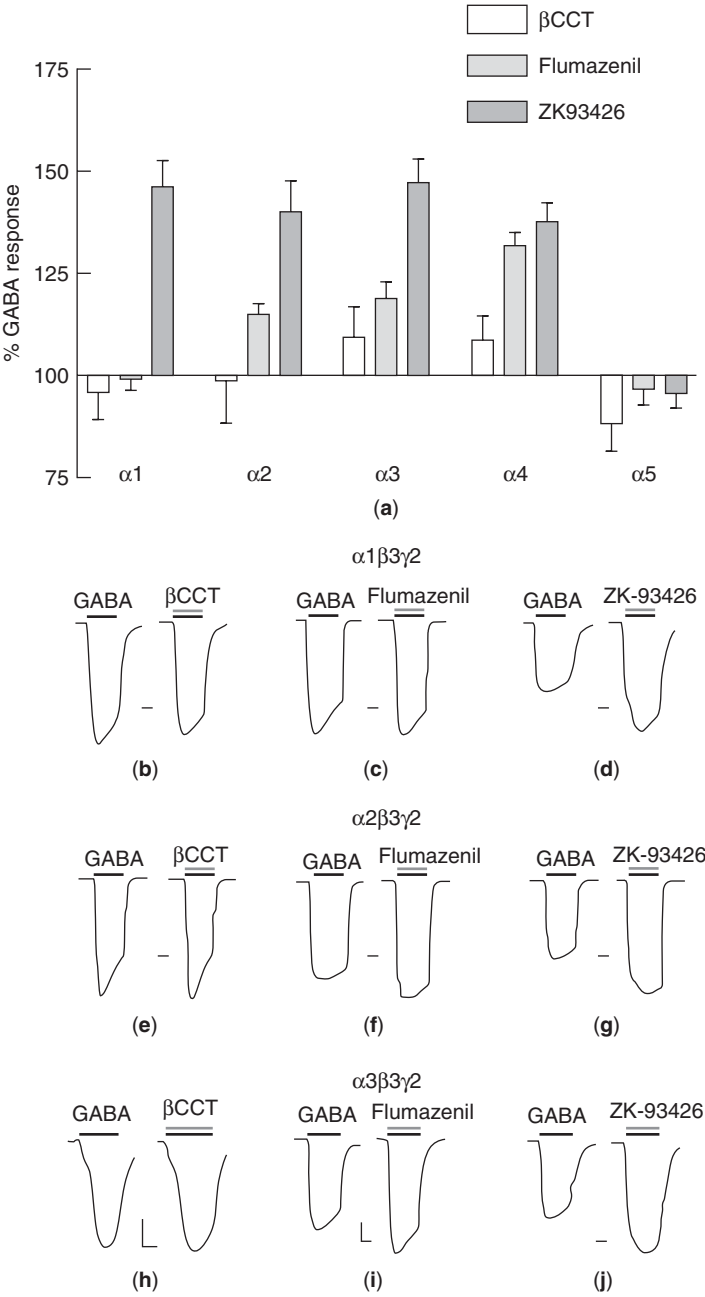
In summary, the efficacy data suggest a qualitatively similar profile for β CCt and flumazenil across recombinant GABA_A receptors bearing the α_1 to α_5 subunits. However, β CCt did not significantly affect GABA currents at the α_1 , α_2 , α_3 , or α_4 (Fig. 14.14) subunits; in contrast, flumazenil increased GABA currents at the α_2 ($p < 0.09$), α_3 ($p < 0.05$), and α_4 ($p < 0.01$) subunits relative to the control condition. The effects of 3PBC mirrored that of flumazenil at the α_2 , α_3 , α_4 , and α_5 subunits compared with the control condition (i.e., GABA alone) ($p < 0.01$) [37]. Hence, while β CCt is relatively GABA neutral (i.e., only a minute GABA potentiation) across all receptor subunits studied, 3PBC and flumazenil are partial agonists at some subunits (α_2 , α_3 , and α_4) and relatively GABA neutral at others (α_1 and α_5). In contrast, however, ZK 93426 produces full agonism at the α_1 to α_4 subunits and GABA-neutral activity at the α_5 subunit. It is possible that the low-efficacy agonist activity across multiple receptor subunits could account for the behavioral pharmacological profile observed with both β CCt and 3PBC in the in vivo behavioral studies noted above.

14.7 EMPLOYING LIGANDS WITH PREFERENTIAL SELECTIVITIES FOR α_1 SUBUNIT CONTAINING GABA_A RECEPTOR AS PHARMACOLOGICAL PROBES TO INVESTIGATE NOVEL ALCOHOL REWARD SUBSTRATES

14.7.1 Ventral Pallidum

Of the potential GABA_A receptors involved in the reinforcing properties of alcohol, evidence suggests the GABA_{A1} subtype within the VP may play an important role in regulating alcohol-seeking behaviors. First, the VP contains one of the highest densities of α_1 subunits in the mesolimbic system [142, 143, 213, 214] (see Fig. 14.15). Second, while a number of unilateral GABAergic projections exist across the reward circuitry from various brain substrates, dense reciprocal GABAergic projections exist from the VP to the NAcc [64, 215–217] (see Fig. 14.16). Third, acute EtOH administration has been reported to selectively enhance the effects of iontophoretically applied GABA in the VP, and these effects are highly correlated with [³H]zolpidem binding (an α_1 subunit selective agonist) [218, 219]. Finally, prior

reports have demonstrated that the VP plays a role in regulating the rewarding properties of psychostimulants and opioids [43, 194, 220]. Together, the above findings suggest a possible role for the VP GABA_A receptors in the euphoric properties of alcohol. We hypothesize that the VP might be functionally relevant in regulating alcohol-motivated behaviors.



14.7.2 Systemic Studies

The initial objective of these studies was to evaluate the selectivity of systemically administered β CCt and 3PBC to decrease responding maintained by EtOH presentation in P and HAD rats. Figure 14.17a shows the effects of β CCt (5–40 mg/kg) in P rats ($N=10$) on a concurrent schedule presentation of EtOH and saccharin when response rates are equated at basal levels [19]. All tested doses of β CCt significantly suppressed EtOH responding. Twenty-four hours post-drug administration the 40-mg/kg dose continued to suppress EtOH responding by 75% of control levels. Further, similar to the effects observed with ZK 93426 and CGS 8216 [166] (nonselective BDZ antagonists), no significant suppression occurred on saccharin responding on day 1 or 24 h post-drug administration (bottom portion

Figure 14.14 Modulation of GABA_A $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, $\alpha_4\beta_3\gamma_2$, and $\alpha_5\beta_3\gamma_2$ receptor subunit combinations expressed in Ltk cells by β CCt (open bars), flumazenil (shaded bars), and ZK 93426 (black bars). A saturating concentration (1–10 μ M) was coapplied over voltage-clamped oocytes along with an EC₅₀ of GABA. (a) Each value is the mean % GABA response \pm SD of at least four separate oocytes. Actions of β CCt, flumazenil, and ZK 93426 on recombinant GABA_A receptor subtypes. Top, current responses of voltage-clamped oocytes expressing GABA_A $\alpha_1\beta_3\gamma_2$ receptors (b) during application of 50 μ M (EC₅₀) GABA alone for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 50 μ M GABA along with 10 μ M β CCt for the duration indicated by the open bar (right trace). (c) Current response of a voltage-clamped oocyte during application of 50 μ M GABA for the duration indicated by the black bar (left trace). Current response from same oocyte subsequently coapplied with 50 μ M GABA along with 1 μ M flumazenil for the duration indicated by the open bar (right trace). (d) Current response of a voltage-clamped oocyte during application of 50 μ M GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 50 μ M GABA along with 10 μ M ZK 93426 for the duration indicated by the open bar (right trace). Center, current responses of voltage-clamped oocytes expressing GABA_A $\alpha_2\beta_3\gamma_2$ receptors (e) during application of 50 μ M (EC₅₀) GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 50 μ M GABA along with 10 μ M β CCt for the duration indicated by the open bar (right trace). (f) Current response of a voltage-clamped oocyte during application of 50 μ M GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 50 μ M GABA along with 10 μ M flumazenil for the duration indicated by the open bar (right trace). (g) Current response of a voltage-clamped oocyte during application of 50 μ M GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 50 μ M GABA along with 10 μ M ZK 93426 for the duration indicated by the open bar (right trace). Bottom, current responses of voltage-clamped oocytes expressing GABA_A $\alpha_3\beta_3\gamma_2$ receptors (h) during application of 30 μ M (EC₅₀) GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 30 μ M GABA along with 10 μ M β CCt for the duration indicated by the open bar (right trace). (i) Current response of a voltage-clamped oocyte during application of 30 μ M GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 30 μ M GABA along with 1 μ M flumazenil for the duration indicated by the open bar (right trace). (j) Current response of a voltage-clamped oocyte during application of 30 μ M GABA for the duration indicated by the black bar (left trace). Current response from the same oocyte subsequently coapplied with 30 μ M GABA along with 10 μ M ZK 93426 for the duration indicated by the open bar (right trace). Scale bars: 5 nA, 10 s. Adapted from June et al., 2003.

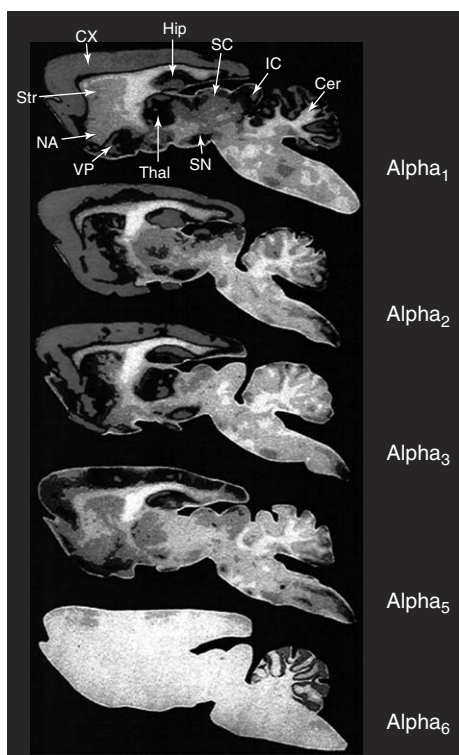


Figure 14.15 Distribution of GABA_A receptor containing subunits α_1 - $\alpha_6\beta_2\gamma_2$ in rat CNS based on immunocytochemistry studies by Turner and colleagues [142]. (See color insert.)

of Fig. 14.17a). Figure 14.17a shows basal operant response rates for EtOH and sucrose were very similar in the HAD rats. The 1- to 10-mg/kg β CCt injections dose dependently suppressed responding maintained by alcohol. The bottom portion of Figure 14.17b shows that β CCt suppressed responding maintained by sucrose only with the 10-mg/kg dose.

Figure 14.18 shows data for P rats following i.p. administration of 1–20 mg/kg of 3PBC. Prior to 3PBC administration the no-injection control (EtOH 201 ± 51 responses; saccharin 153 ± 23 responses) and the Tween-20 vehicle (EtOH 191 ± 42 ; saccharin 161 ± 41) conditions were similar. Hence, these data were pooled and used to compare against the drug treatment. The black bars of Figure 14.18 show that 3PBC produced a significant dose-related reduction on EtOH-maintained responding, reducing responding by as much as 35–84% of control levels. The hashed bars of Figure 14.18 show that, in contrast to the effects observed on alcohol responding, the lower 3PBC doses (1–10 mg/kg) markedly and significantly elevated responding maintained by saccharin, while the 20-mg/kg dose markedly and significantly suppressed responding. Together, these data show that, given systemically, β CCt and 3PBC are capable of selectively reducing EtOH-maintained responding. The data also demonstrated that across two alcohol-selected rat lines, β CCt can function as an antagonist of EtOH-seeking behaviors and not suppress responding for a palatable noncaloric or caloric solution given concurrently with EtOH.

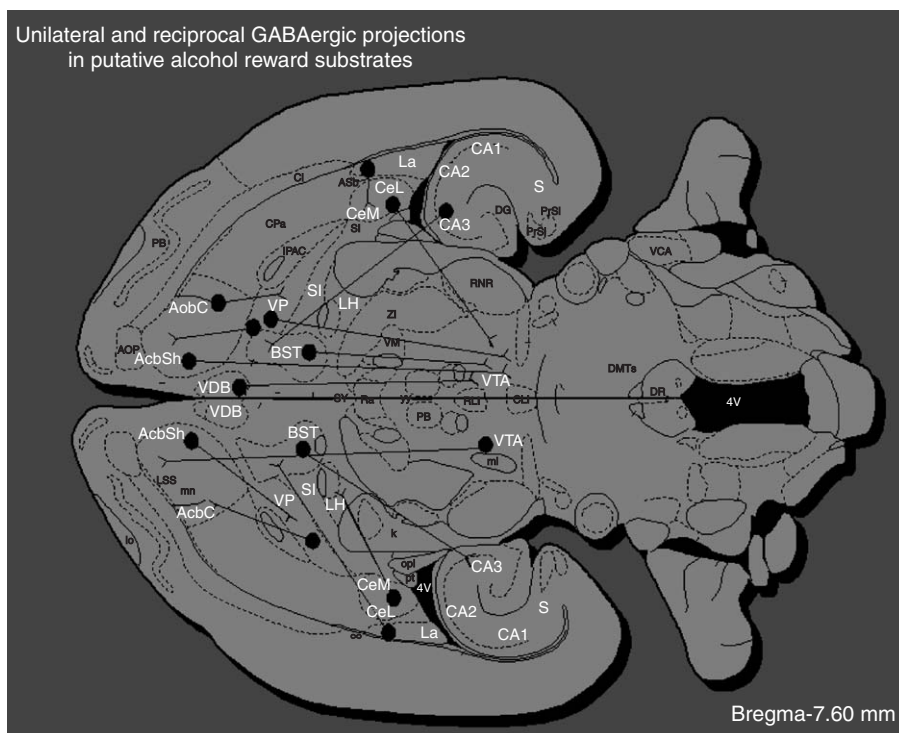


Figure 14.16 Illustration of both unilateral and reciprocal GABAergic projections in putative alcohol reward substrates based on histological mapping studies [64, 87, 88, 94, 95, 215–217]. (See color insert.)

14.7.3 Microinjection Studies

The second objective employing β CCt and 3PBC was to *directly* test the hypothesis that the GABA_{A1} receptor within the VP plays an important role in regulating alcohol-seeking behaviors. It should be recalled that a substantial number of α_1 -subunit-containing GABA_A receptors exist within the VP, while exceedingly low or nondetectable levels of the other α subunits (e.g., α_2 , α_3 , α_4 , α_5 , and α_6) are found within the VP [142, 143, 213, 214]. Figure 14.19a shows behavioral data for P rats bilaterally infused with β CCt (5–40 μ g) in the VP compared with the no-injection baseline (i.e., BL) and the artificial cerebral spinal fluid (aCSF) control conditions. β CCt dose dependently reduced EtOH-maintained responding relative to control conditions. In contrast, β CCt was without effect on responding maintained by saccharin (Fig. 14.19a). Finally, β CCt failed to alter responding maintained by EtOH or saccharin following infusion into the NAcc or caudate putamen (CPu) area (Fig. 14.19b). Hence, β CCt displayed both reinforcer and neuroanatomical specificity.

Figures 14.19c–e show data for HAD rats following administration of β CCt (5–40 μ g). The HAD rats were trained to lever press for alcohol only; HAD rats do not lever press for alcohol in significant quantities when concurrently presented with highly palatable reinforcers [4]. To further substantiate the neuroanatomical specificity of the GABA_{A1} receptor in alcohol-related behaviors, HAD rats received a unilateral implant in the VP and a second implant in either the CPu or NAcc.

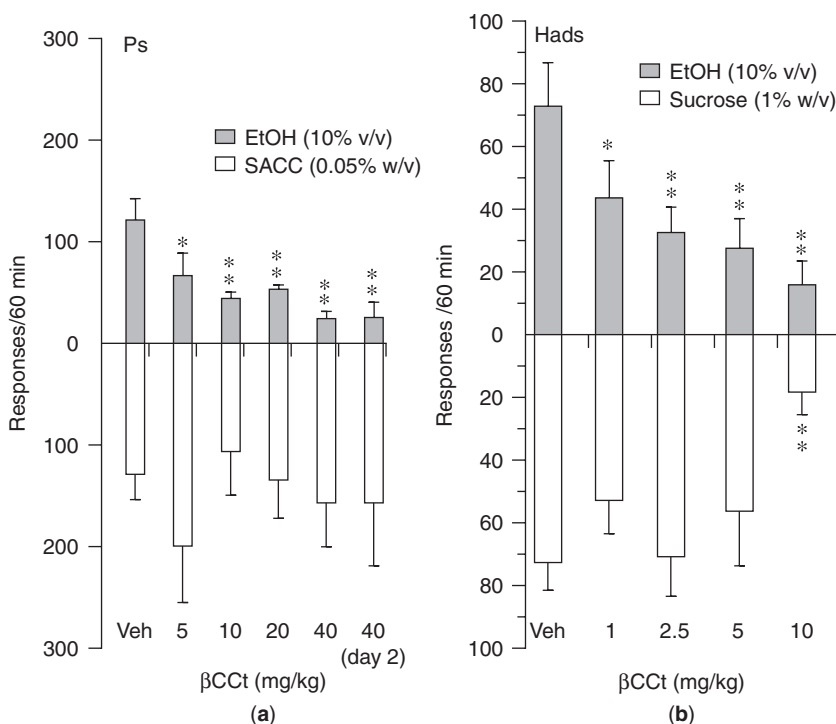


Figure 14.17 Dose-response of systemic β CCt injections (i.p.) in (a) P (Ps) (5–40 mg/kg) and (b) HAD-1 (Hads) (1–10 mg/kg) rats. P rats ($N=11$) performed under a concurrent FR4 schedule for EtOH (10% v/v) and saccharin (0.05% w/v). HAD-1 rats ($N=11$) performed under an alternate-day access paradigm, wherein they received EtOH (10% v/v) on day 1 and sucrose (1% w/v) on day 2. At 15 min after the i.p. injections, rats were placed in the operant chamber to lever press for a 60-min session. (**) $p<0.01$ and (*) $p<0.05$ vs the control condition values by ANOVA and post hoc Newman–Keuls test. Bars represent means \pm SEM. Adapted from June et al., 2003.

Figures 14.19c–e show rates of responding maintained by EtOH following unilateral microinjection of the 0.5- to 7.5- μ g doses of β CCt into the VP of HAD rats. Compared with the aCSF control condition, β CCt dose dependently reduced EtOH responding. In addition, 24 h post-drug administration the 2.5- to 7.5- μ g doses continued to reduce responding by as much as 54–63% of control levels. In contrast, unilateral infusions of β CCt into the CPu/NAcc areas were completely ineffective in altering alcohol-maintained responding (Figs. 14.19d,e).

A study by Harvey et al. [37] evaluated the efficacy of bilaterally administered 3PBC in the VP or NAcc/CPu employing a similar experimental design as June et al. [19]. 3PBC (0.5–40 μ g) was found to dose dependently and selectively reduce alcohol-maintained responding relative to the control condition (see Fig. 14.20). Only the 40- μ g dose nonsignificantly reduced saccharin responding. To determine the neuroanatomical specificity of the VP α_1 subunit modulation of alcohol-maintained responding, Harvey et al. [37] evaluated the capacity of 3PBC to reduce alcohol-motivated behaviors in the NAcc/striatum, a locus reported to be devoid of the α_1 receptor

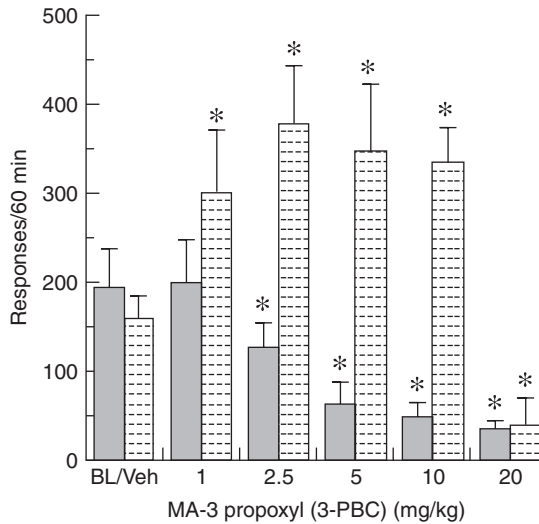


Figure 14.18 Dose–response of systemic (0.0–20 mg/kg; $n = 13$) injections of 3PBC on a concurrent FR4 schedule for EtOH (10% v/v) and saccharin (0.05% w/v) responding during the 1-h operant session. (*) $p < 0.05$ versus the control condition values by ANOVA and post hoc Newman–Keuls test. Error bars indicate means \pm SEM. The two control conditions were pooled in the systemic group and compared against the drug treatment conditions. BL, Baseline; Veh, vehicle control. Adapted from Harvey et al., 2002.

subunit [141–143, 214]. Similar to the β Cct treatments, responding maintained by EtOH and saccharin (Fig. 14.20b) following 3PBC in the NAcc/CPu locus was not altered. Hence, these data indirectly confirm the significant topography of the GABA_{A1} receptor in the pallidal area of the P rats for EtOH-maintained responding [213, 214].

Taken together, these data show that β Cct and 3PBC in the VP are capable of selectively reducing EtOH-maintained responding with little if any effect on responding for a palatable saccharin/sucrose reinforcer. Since both β Cct and 3PBC are moderately selective for the GABA_{A1} and a high density of these receptors are located in the VP, it is reasonable to hypothesize that the attenuation seen by these agents are mediated at least in part via the GABA_{A1} receptors. Further, some degree of neuroanatomical specificity was also observed since neither β Cct nor 3PBC altered EtOH responding when infused into the NAcc or CPu.

14.8 ORAL ADMINISTRATION OF β CCT OR 3PBC PRODUCES PROLONGED REDUCTION ON ALCOHOL SELF-ADMINISTRATION: DIRECT COMPARISON WITH OPIOID RECEPTOR ANTAGONIST NALTREXONE

To evaluate potential pharmacotherapies for alcohol dependence in rodents, it is necessary to determine their capacity to reduce alcohol drinking via the oral route. Employing the oral gavage route of administration, June et al. (unpublished)

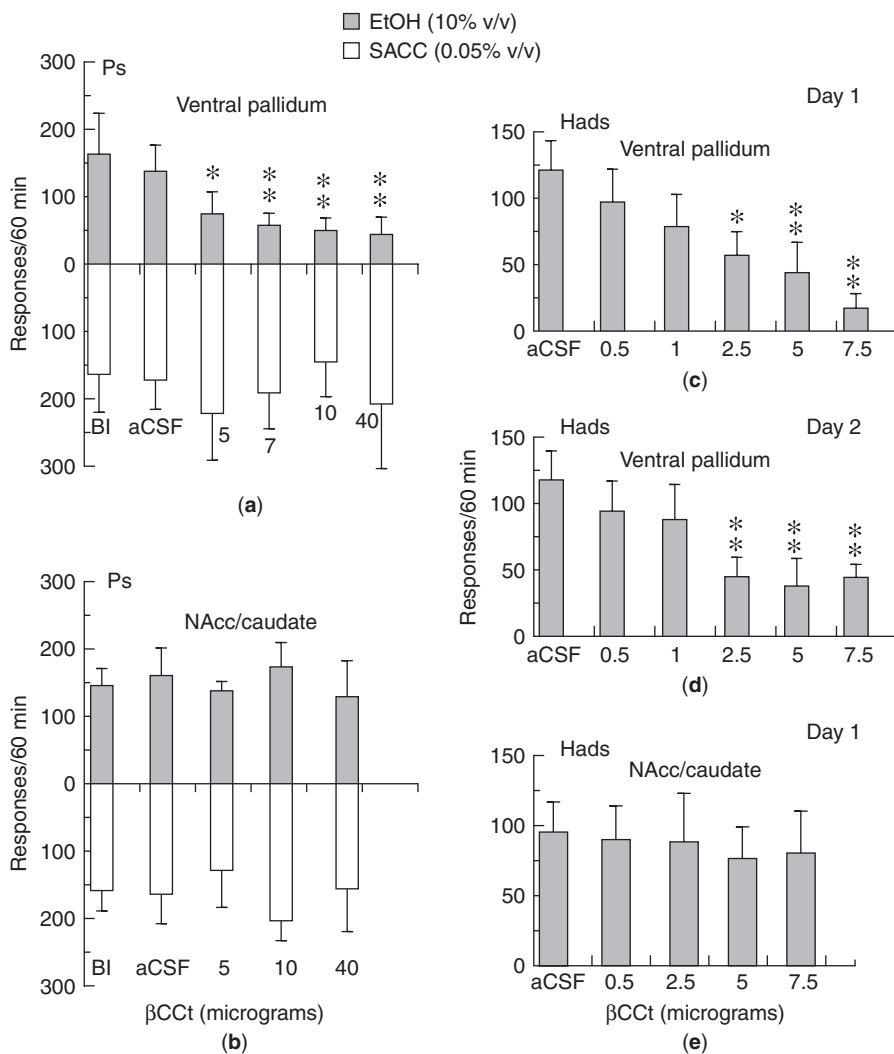


Figure 14.19 (a) Performance of female P rats ($n=11$) on a concurrent FR4 schedule for EtOH (10% v/v) and saccharin (0.05% w/v) following bilateral infusions of β CCt (0.0–40 mg) in the VP. (b) Performance of control female P rats ($n=7$) on a concurrent FR4 schedule for EtOH (10% v/v) and saccharin (0.05% w/v) following bilateral infusions of β CCt (0.0–40 mg) in the NAcc/CPu areas. (c, d) Performance of female Had rats ($n=9$) on an FR4 schedule for EtOH (10% v/v) following unilateral infusions of β CCt (0.0–7.5 mg) in the VP on the first day of infusion and 24 h post-drug administration. (e) Performance of the same female Had rats in (c) ($n=9$) on an FR4 schedule for EtOH (10% v/v) following unilateral infusions of β CCt (0.0–7.5 mg) in the NAcc/CPu areas on the first day of infusion. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the baseline (BL) and aCSF conditions using post hoc Newman–Keuls tests. Error bars indicate means \pm SEM. Adapted from June et al., 2003.

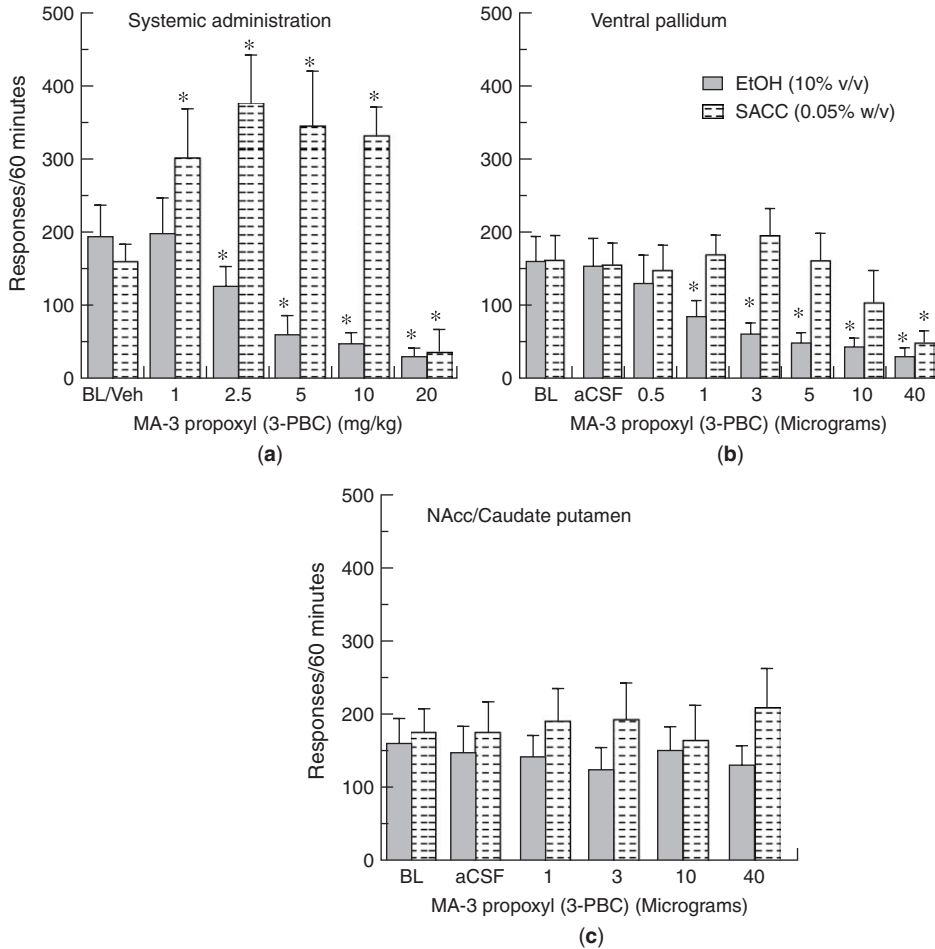


Figure 14.20 Dose–response bilateral infusions of 3PBC (0.5–40 μ g) in the VP (a; $n = 12$) and NAcc/CPu (neuroanatomical control loci) (b; $n = 7$) on a concurrent FR4 schedule for EtOH (10% v/v) and saccharin (0.05% w/v) responding during the 1-h operant session. (*) $p < 0.05$ versus the control conditions values by ANOVA and post hoc Newman–Keuls test. Error bars indicate means \pm SEM. BL, Baseline; Veh, vehicle control. Adapted from Harvey et al., 2002.

evaluated the actions of β CCt and 3PBC (0–75 mg/kg) on alcohol-motivated responding. The nonselective opioid antagonist naltrexone was used as a reference agent, since it is well established as an alcohol antagonist that reduces both alcohol cravings and intake [221]. Figure 14.21 shows data for naltrexone on day 1, 30 min after drug administration. Naltrexone produces clear dose-dependent reduction on alcohol responding on day 1; however, no effects on alcohol responding were observed on day 2. Figure 14.22 shows that, similar to naltrexone, dose-dependent reductions were observed on alcohol responding on day 1, 30 min following β CCt administration; however, in contrast to naltrexone, marked reductions on alcohol drinking were still detectable 24 h postdrug. 3PBC also produces dose-dependent reductions on alcohol responding on day 1, and similar to β CCt, the marked reductions on alcohol drinking were still apparent 24 h later (Fig. 14.23). At the

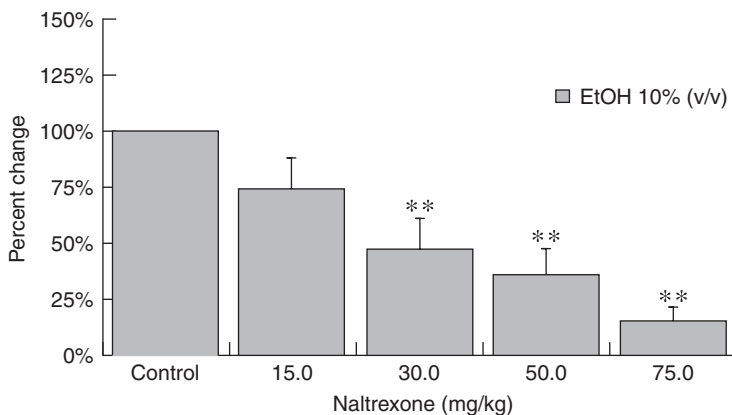


Figure 14.21 Responding for 10% EtOH (v/v) expressed as a percent of baseline line following oral administration of naltrexone (15, 30, 50, 75 mg/kg); **, $p < 0.01$.

highest tested dose (e.g., 75 mg/kg) neither β CCt nor 3PBC altered responding maintained by sucrose; however, naltrexone profoundly reduced sucrose responding. Together, these data show that both β CCt and 3PBC are as effective as naltrexone in reducing alcohol drinking following acute administration. However, β CCt and 3PBC are far superior to naltrexone in their duration of action on alcohol responding, with suppression observed for at least 24 h postadministration. In addition, unlike naltrexone, neither β CCt nor 3PBC reduced sucrose (a highly palatable caloric reinforcer) responding. These findings suggest that β CCt and 3PBC are excellent candidates for reducing alcohol intake and cravings in humans and are not likely to nonselectively reduce other consummatory behaviors such as feeding and drinking.

14.8.1 Oral Efficacy of β CCt and 3PBC in Reducing Anxiety in Alcohol-Preferring Rats

The in vitro data presented above suggest that both β CCt and 3PBC produce agonist activity at some GABA_A receptors while exerting GABA neutral/antagonist activity at others. In many instances, in vitro pharmacology does not extrapolate to whole-animal pharmacology. However, given that β CCt and 3PBC produced a sustained reduction in alcohol drinking behavior, an added advantage of these agents would be a demonstrated effectiveness in reducing anxiety. It is well established in addiction psychiatry that anxiety and alcohol abuse are comorbid disorders [222]. Furthermore, it is possible that anxiety may predispose subjects to initiate alcohol drinking behaviors [223, 224]. Hence, the oral effectiveness of β CCt and 3PBC was evaluated in reducing anxiety (assessed using the elevated-plus maze) in alcohol-preferring rats. Gavage of β CCt and 3PBC was indeed effective in reducing the anxiety in P rats (Fig. 14.24) with β CCt also effective in reducing anxiety in HAD rats. Compared with the NP rat, the P rat is more “anxious” in the plus maze assay [224]. Thus, this behavioral assay is well suited for the assessment of novel anxiolytics in P rats. Taken together, the oral effectiveness of β CCt and 3PBC as anxiolytics combined with their oral efficacy in reducing alcohol drinking behavior makes both of these compounds

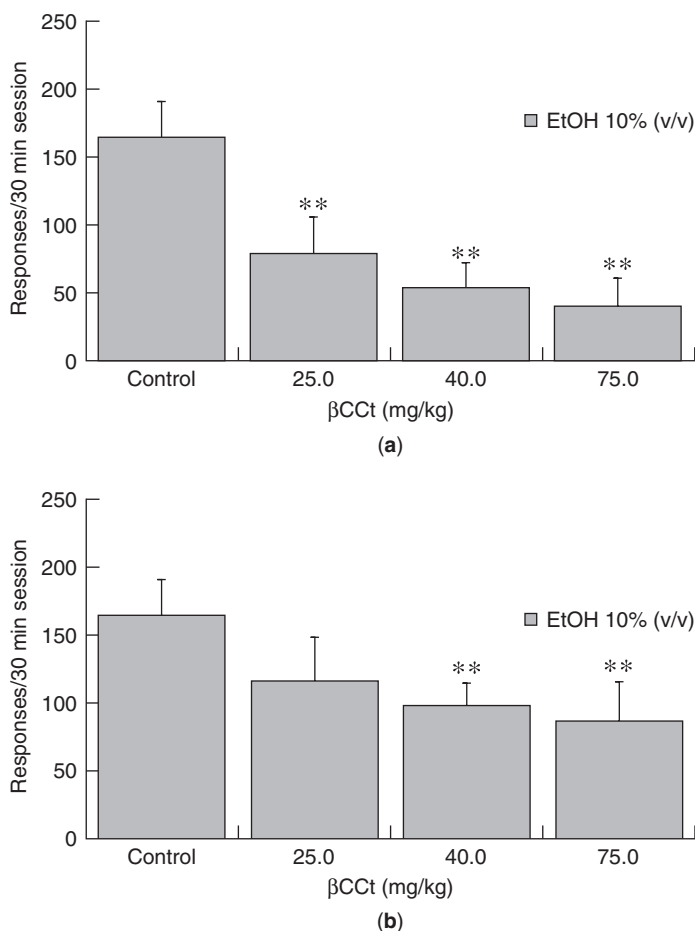


Figure 14.22 Responding for 10% EtOH (v/v) following oral administration of β CCt (25, 40, 75 mg/kg) (a) and 24 h postadministration (b); **, $p < 0.01$.

ideal as prototype pharmacotherapeutic agents in reducing alcohol drinking behaviors in humans.

14.9 EMPLOYING LIGANDS WITH PREFERENTIAL SELECTIVITIES FOR α_5 -SUBUNIT-CONTAINING GABA_A RECEPTORS AS PHARMACOLOGICAL PROBES TO INVESTIGATE NOVEL ALCOHOL REWARD SUBSTRATES: CA1 AND CA3 HIPPOCAMPUS

While GABA_{A5} receptors are minor constituents of the total GABA_A receptor pool, immunocytochemical, in situ hybridization, and radioligand binding studies show that the CA1, CA2, and CA3 fields are enriched in this subunit compared with other brain areas [141, 143, 225]. Only, low or nondetectable levels of other subunits can be localized within the hippocampus. The CA1 and CA3 hippocampal fields are

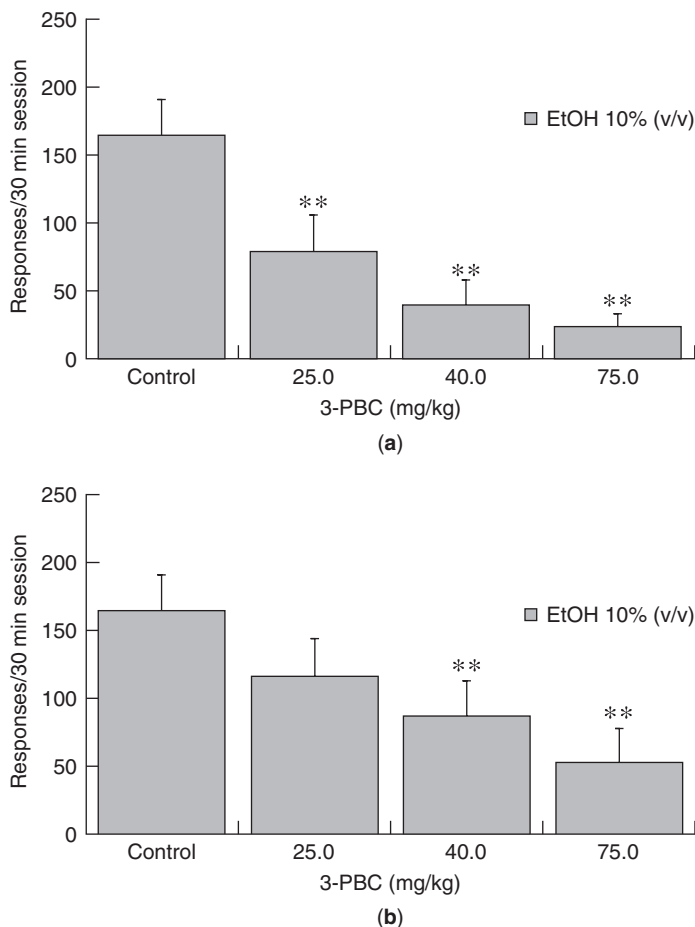


Figure 14.23 Responding for 10% EtOH (v/v) following oral administration of 3PBC (25, 40, 75 mg/kg) (a) and 24 h postadministration (b); **, $p < 0.01$.

particularly interesting candidate sites for the study of alcohol-motivated behaviors since projections from the CA1 and CA3 fields, via the subiculum, innervate several putative EtOH reward substrates [e.g., NAcc, amygdala, bed nucleus of the stria terminalis (BNST), hypothalamus, olfactory tubercle] [215, 226, 227].

RY 023 (*tert*-butyl-8-(trimethylsilyl) acetylene-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5a] [1,4]-benzodiazepine-3-carboxylate) (Table 14.2) is one of a series of 8-substituted imidazobenzodiazepine inverse agonists [145, 228, 229] developed from the anti-EtOH agent RO 15-4513 [132]. RY 023 exhibits both high binding affinity ($K_i \sim 2.7$ nM) and selectivity (~ 75 -fold) at recombinant GABA_A receptors composed of $\alpha_5\beta_2\gamma_2$ subunits [229] (see Table 14.1). RY 023 acts as a negative modulator at the $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, and $\alpha_5\beta_3\gamma_2$ receptor subunits, inhibiting GABA-evoked current responses of voltage-clamped *Xenopus* oocytes by approximately 40–55%. The efficacy of the imidazobenzodiazepines RO 15-4513 and RO 15-1788 (flumazenil) were also examined at the α_1 , α_2 and α_5 subunits. These parent compounds were

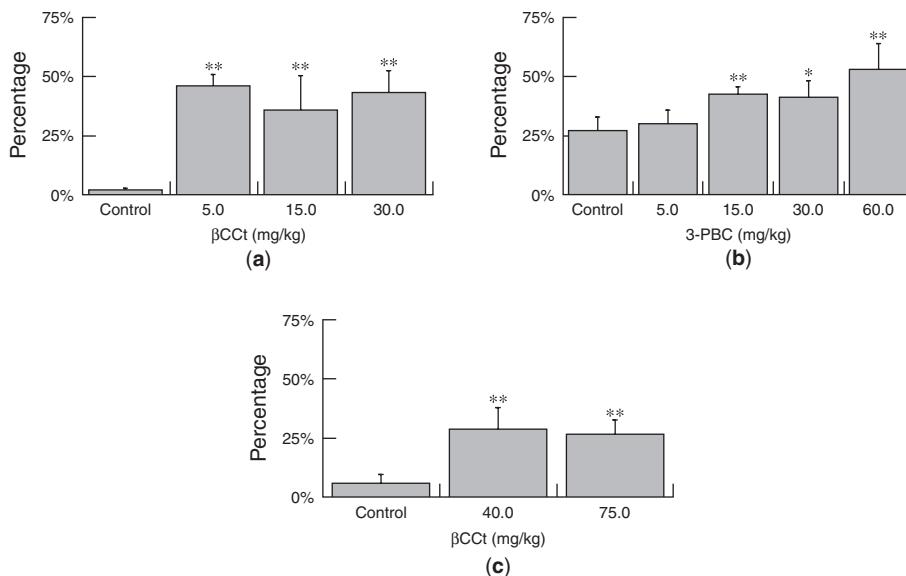


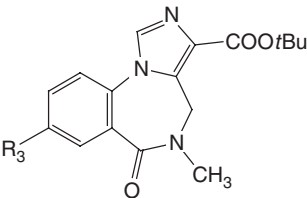
Figure 14.24 Percent of total time spent in open arms of an elevated-plus maze following oral administration of β CCt (5, 15, 30 mg/kg) (a) and 3PBC (5, 15, 30, 60 mg/kg) (b) in P rats and (40 and 75 mg/kg) in HAD rats (c); (*) $p < 0.05$ and ** $p < 0.01$.

selected since June et al. [18] were interested in evaluating the degree to which modification at position 8 (which produced the α_5 selectivity) [18, 228, 229] would change the imidazobenzodiazepine's capacity to modulate GABA at specific receptor subtypes. RO 15-4513 was also selected to compare the intrinsic activity of a reference alcohol antagonist (see [132, 230, 231]) with RY 023. RO 15-4513 produced a very modest inhibition of GABA current at the α_1 and α_2 subunits ($86 \pm 3\%$ and $93 \pm 1\%$ control response, respectively), while exhibiting no efficacy at the α_5 subunit ($99.5 \pm 4.1\%$ control response). RO 15-1788 (flumazenil), the competitive antagonist [232–234], acted as a modest positive modulator at the α_2 subunit ($115 \pm 4\%$ control response) and exhibited no efficacy at either the α_1 or α_5 subunit.

Taken together, these data reveal several important potency and efficacy differences between RY 023 and the alcohol antagonist RO 15-4513. First, the selectivity of RY 023 for the α_5 receptor subunit is higher than RO 15-4513 (see Table 14.1). Second, the magnitude of GABAergic reduction with RY 023 is far greater than that of RO 15-4513 at the α_1 , α_2 and α_5 receptor subunits [18]. Third, RO 15-4513 exhibits an efficacy profile similar to that of RO 15-1788 at the α_2 and α_5 subunits [18]. Hence, it is possible that the antialcohol properties of the two inverse agonists may be differentially regulated (see [123, 125]).

June and colleagues [18] tested the hypothesis that α_5 subunits of the CA1 and CA3 hippocampal fields would regulate EtOH-motivated behaviors in the P rat (also see [123, 125]). To accomplish this, the actions of bilateral and unilateral micro-injections of RY 023 in the CA1 and CA3 hippocampal fields were evaluated for their capacity to reduce EtOH-maintained responding. The degree of neuroanatomical specificity was examined using both bilateral and unilateral control injections into the

TABLE 14.2 Affinities of 5,6-Dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5*a*] [1,4]-benzodiazepine-3-carboxylic acid *tert*-Butyl Esters for $\alpha_x\beta_3\gamma_2$ ($x = 1-3, 5, 6$) Benzodiazepine Receptor Isoforms



		K ₁ (nM) ^a					
Ligand	R ₈ ^b	α ₁	α ₂	α ₃	α ₅	α ₆	α ₁ /α ₅
14	Cl	17.3	21.6	29.1	0.65	4	26.6
15	Br	11.4	10.7	9.2	0.47	9.4	24.3
16	I	9.7	11.2	10.9	0.38	4.6	25.5
17	OH	1.50	NA	0.53	0.14	6.89	10.7
18	OCH ₃	6.74	NA	7.42	0.293	8.28	23.0
19	N(CH ₃) ₂	13.1	NA	38.1	0.78	118	16.8
20	X	5.8	NA	169	9.25	325	0.63
21	Y	6.44	NA	148	4.23	247	1.5
22	N=N ⁺ =N ⁻	7.25	NA	5.66	0.3	5.25	24.3
23	NCS	17.1	33.7	50	2.5	30.7	6.8
24	NO ₂	12.8	49.8	30.2	3.5	22.5	3.7
25	Et	14.8	56	25.3	1.72	22.9	8.6
26	C≡C-H	26.9	26.3	18.7	0.4	5.1	67.3
27	C≡C-Si(CH ₃) ₃	197	143	255	2.61	58.6	75.5
28	C≡CCH ₂ Si(CH ₃) ₃	275.0	387.0	337.0	23.0	301.0	12.0

Source: Adapted from Huang et al., 1998.

^aData shown here the means of two determinations which differed by less than 10%. NA, data not available.

^bX, *N*-tetrahydropyrrole; Y, *N*-hexahydropyridine.

NAcc and VTA. Unlike the hippocampal fields, these brain areas possess higher levels of α_2 - and α_1 -subunit expression, respectively [141–143]. The specificity of RY 023 on consummatory responding was evaluated by determining the effects of RY 023 in P rats whose response rates for EtOH (10% v/v) and saccharin solutions (0.05% w/v) were similar at basal levels.

Figure 14.25a shows rates of responding maintained by EtOH (upper panel) following microinjection of the 1.5- to 20- μ g doses of RY 023 into the CA1 (dorsal and CA3 (ventral) hippocampus. RY 023 produced a dose-related suppression on EtOH-maintained responding. The lower panel shows that RY 023 selectively reduced EtOH responding, reducing saccharin responding only at the highest tested dose (20 μ g). When ZK 93426 was administered immediately prior to RY 023, it attenuated the RY 023-induced suppression on EtOH-maintained responding, suggesting the suppression was mediated via actions at the BDZ site of the GABA

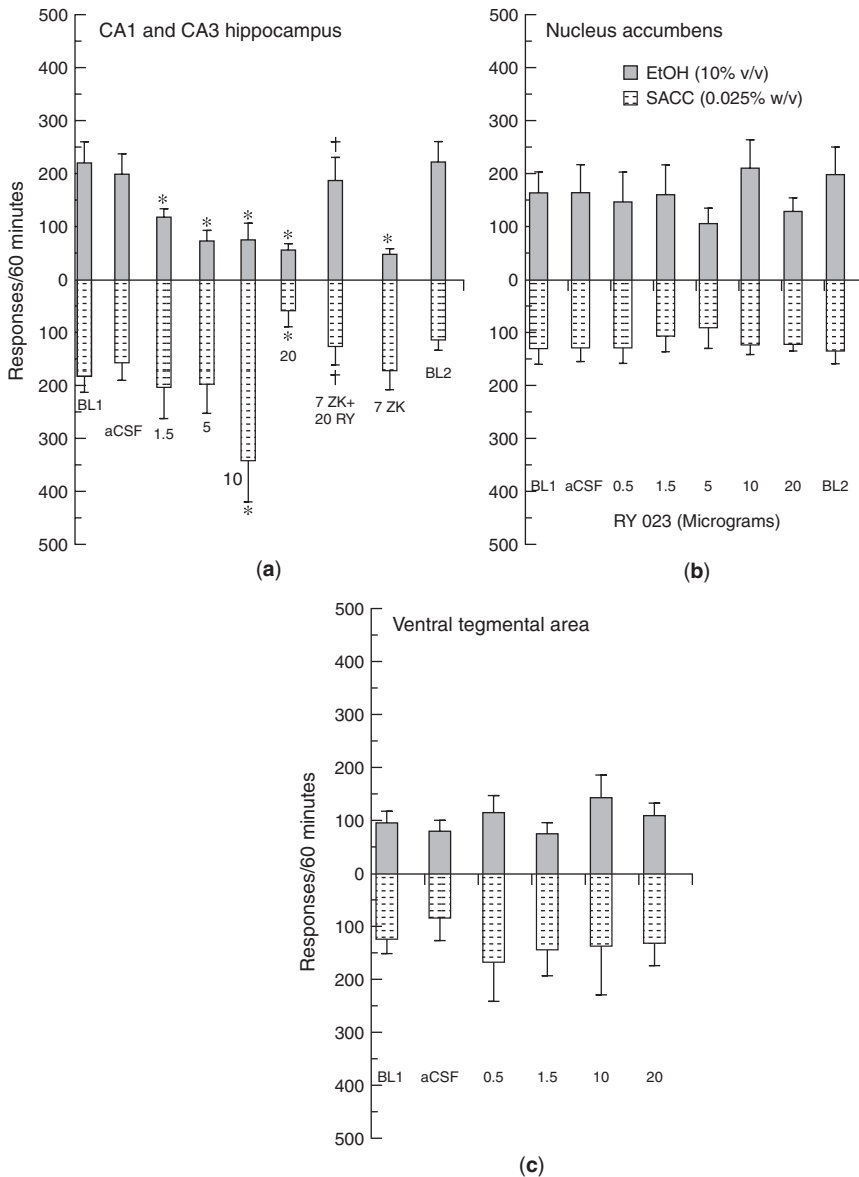


Figure 14.25 Dose–response of bilateral infusions of RY (0.0–20 µg) in the hippocampus ($n=9$) (a), NAcc ($n=9$) (b), and VTA ($n=10$) (c) on a concurrent FR4 schedule for EtOH (10% v/v) and saccharin-maintained (0.025% w/v) (SACC) responding. Immediately after the microinfusions, rats were placed in the operant chamber to lever press for a 60-min session. (*) $p < 0.05$ versus the no-injection control conditions (BL1 and BL2) and aCSF control condition values by ANOVA and post hoc Newman–Keuls test ($n=9$). Error bars represent \pm SEM. (†) $p < 0.01$ versus the 20 µg RY alone condition by ANOVA and post hoc Newman–Keuls test ($n=9$). ZK, the competitive BDZ antagonist, completely reverses the suppression by RY on EtOH and saccharin-motivated responding. RY was without effect on EtOH or saccharin-maintained responding in the NAcc and VTA. Adapted from June et al., 2001.

receptor complex Figures 14.25b,c show that, in addition to reinforcer selectivity, RY 023 was anatomically selective. Specifically, bilateral infusion into the NAcc or VTA was without effect on responding maintained by EtOH (upper panel) and saccharin (lower panel). Unilateral infusions of RY 023 produced a clear dose-dependent suppression on EtOH-maintained responding. With the exception of the highest tested dose (40 μ g), reinforcer selectivity was also observed, as was neuroanatomical selectivity, with no effects on EtOH-maintained responding observed in the NAcc or VTA. RY 023 also failed to alter saccharin-motivated behaviors in the NAcc and VTA.

The above findings provide the *first* demonstration that GABA_A receptors containing α_5 subunits in the hippocampus play a critical role in regulating alcohol-seeking behavior [18]. The findings are supported by recent research with outbred rats [125] and a related α_5 subunit selective ligand, RY 024, in P rats [123]. Finally, previous work has shown that an α_5 agonist substitutes for alcohol in primate drug discrimination studies [235]. Thus, these findings suggest that the hippocampus/hippocampal projection sites may be functionally relevant in regulating EtOH-motivated behaviors.

The precise GABA–hippocampal pathway(s) in which the GABA_{A5} selective ligands modify EtOH reinforcement/related responding is unknown; it has been hypothesized that GABA_A BDZ neuroanatomical circuits within the hippocampus may initiate activation of underlying DA substrates in the mesoaccumbens circuitry to contribute to the reinforcing properties of EtOH [18]. The functional role of conditioning stimuli in the onset and maintenance phases of alcohol-motivated drinking may also be important. It should be recalled that BDZs have long been proposed to modulate learning and memory processes via the hippocampus [236]. We conclude that the GABA–hippocampal pathway may represent an “extension” of the mesolimbic EtOH reward circuitry as has been proposed by Koob and colleagues [52]. Thus, the hippocampus may be an important *target* in the development of potential pharmacotherapies for alcohol addiction and dependence. The *Xenopus* oocyte studies demonstrated that the capacity of BDZs to attenuate EtOH-motivated responding was not directly related to their intrinsic efficacy; rather, their selectivity and differential potency to attenuate EtOH-seeking behaviors appear to be more related to their affinity and selectivity at different GABA_A-containing receptor subunits. However, it is possible that the *in vitro* efficacy observed in the *Xenopus* oocyte assay may not reflect the *in vivo* efficacy in an animal model of alcoholism following BDZ administration.

14.10 SUBUNIT SELECTIVITY VERSUS INTRINSIC EFFICACY

The rank-order potencies of BDZ site ligands to attenuate EtOH intake are not correlated with either rank-order potencies to inhibit GABA $^{36}\text{Cl}^-$ conductance or enhance ^{35}S -*t*-butylbicyclopophosphorothionate (TBPS) binding [237]. While the ^{36}Cl flux and TBPS binding assays in brain tissues employ heterogenous subunit populations, with the resulting value obtained representing an “average efficacy,” the *Xenopus* oocyte system permits efficacy to be determined at any different subunit–complementary DNA (cDNA) combinations [140, 211, 212]. In the *Xenopus* system RO 15-4513 was essentially GABA neutral at the α_2 - and α_5 -subunit-containing

receptors and slightly GABA negative at the GABA_{A1} receptor. These data are in agreement with previous oocyte studies [29, 211, 212] and work by Wong and Skolnick [238] employing the “GABA shift” assay. The findings with RO 15-4513, however, contrast those with RY 023 and ZK 93426, where the GABA-evoked current is negatively and positively modulated, respectively, at the α_1 and α_2 receptor subunits. Nevertheless, despite the three different intrinsic activity profiles, each ligand was highly effective in attenuating EtOH-motivated behaviors [78, 156, 161, 166, 237]. Thus, while both efficacy and subunit selectivity may interact to effectively alter EtOH-motivated behaviors, the evidence in this review strongly suggests that subunit selectivity may be the critical factor in determining the capacity of a ligand to function as an effective alcohol antagonist. Several investigators suggest that efficacy not only is dependent on subunit composition but also is actually defined by it [145, 162a, 238, 239].

14.11 CONCLUSIONS

Research on the substrates and circuitry regulating alcohol self-administration has experienced enormous growth over the past decade. The primary purpose of this review was to provide the reader with relatively new research findings that have been critical in the delineation of novel brain reward substrates that can be targeted in the potential development of pharmacotherapeutic prototypes for alcohol addiction and dependence in humans. Alcohol-dependent individuals represent a heterogeneous group [19, 240–242]. Hence, it is unlikely that a single pharmacological treatment will be effective for all alcoholics. To this end, a better understanding of the neuromechanisms which regulate alcohol-seeking behaviors and the design of clinically safe and effective drugs that reduce alcohol addiction and dependence remain a high priority in alcoholism research [243–245].

In this review, we presented data implicating novel substrates and circuitry within the dopaminergic and GABAergic systems in alcohol reward. Prior to presenting these data, however, the significance of an optimal animal model of alcoholism and its integration with appropriate behavioral and neuroanatomical paradigms to accurately interpret findings obtained from alcohol self-administration studies were discussed. Relatively new data that implicated brain loci such as the VP, BST, and LH in regulating alcohol-motivated behaviors within the DA systems were also presented. Our conceptual framework was based on the premise that GABA mediates its actions on alcohol self-administration via interaction with the DA system [19, 37]. Indeed, current studies are underway in our laboratory showing that in many cases this DAergic regulation of alcohol intake can in fact be modulated via GABA receptors in other mesolimbic brain reward areas [104]. Finally, June et al. presented compelling data showing that, using a multidisciplinary approach (e.g., medicinal chemistry, molecular biology, behavioral pharmacology, neuroanatomy), it was indeed feasible to exploit the GABAergic system in the potential development of pharmacotherapies for alcohol addiction and dependence in humans (but see [245]). The primary GABA_A receptor candidates in these efforts were the α_1 and α_5 receptor subunits. However, the research efforts exploiting the α_1 receptor subunit demonstrating the oral effectiveness of β CCt and 3PBC as anxiolytics, combined with their oral efficacy in reducing alcohol drinking behavior, are compelling. This seminal

research on β CCt and 3 PBC should set the stage for further investigation in the coming years for evaluation of these agents as prototype pharmacotherapeutic agents in reducing alcohol drinking behaviors in human alcoholics.

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15

NICOTINE

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15.1	Overview	535
15.2	Pharmacology of Nicotine	536
15.2.1	Overview	536
15.2.2	Nicotinic Acetylcholine Receptors	537
15.2.3	Nicotine Reinforcement Neurosubstrates	537
15.2.4	nAChR Functional Adaptations	540
15.2.5	Nicotine Withdrawal Neurosubstrates	541
15.2.6	Pharmacokinetics	543
15.2.6.1	Absorption	544
15.2.6.2	Distribution	544
15.2.6.3	Metabolism and Elimination	545
15.3	Pharmacological Treatment of Nicotine Dependence and Withdrawal	545
15.3.1	Overview	545
15.3.2	Nicotine Replacement Medications	546
15.3.2.1	Transdermal Nicotine Patch	546
15.3.2.2	Acute Delivery Systems	547
15.3.3	Nicotinic Pharmacotherapies	548
15.3.3.1	Nicotinic Partial Agonists	548
15.3.3.2	Nicotinic Antagonists	549
15.3.4	Nonnicotinic Pharmacotherapies	549
15.3.4.1	Bupropion	549
15.3.4.2	Tricyclic Antidepressants	550
15.3.4.3	Cannabinoid Antagonists	550
15.3.4.4	Opioid Antagonists	550
15.3.5	Nicotine Pharmacology Implications for Public Health Policy	551
	Acknowledgments	552
	Disclosures	552
	References	552

15.1 OVERVIEW

Nicotine is the drug delivered upon tobacco use that defines the dependence and withdrawal syndromes as recognized by the World Health Organization (WHO) and

American Psychiatric Association (APA) [1, 2]. Many other potentially psychoactive substances are present in tobacco and/or the smoke of burned tobacco [3], but as concluded by Lewin in the 1920s, “the decisive factor in the [psychic] effects of tobacco, desired or undesired, is nicotine” [4, p. 256]. As a pharmacological agent with diverse and powerful neuronal effects and an important historical role in the development of neuroscience research strategies even before the term *neuroscience* was used, nicotine is fascinating to study.

Nicotine itself is not without toxicity, but at doses typically ingested by tobacco users, its direct contribution to disease is comparatively small relative to the more than 60 carcinogens, carbon monoxide, and many lung and cardiovascular toxins produced by tobacco cigarette smoking. Its role in tobacco-caused disease is primarily to sustain high and persistent levels of toxic tobacco smoke exposure. Thus, most tobacco-caused disease may be considered side effects of nicotine dependence. From a public health perspective, then, nicotine is critical in the scope and persistence of a global tobacco epidemic that is so devastating that it led to the first-ever international treaty negotiated by the WHO, put into force in 2005 [5]. This treaty, in turn, has already begun to drive additional research on nicotine and tobacco as part of its strong advocacy of a public health-driven demand reduction approach to controlling tobacco-caused disease.

Nationally, cigarette smoking has been cited as “the leading cause of preventable death in the United States” and is associated with profound health-related and financial costs [6, 7]. In terms of health costs, cigarette smoking causes approximately 442,398 premature deaths yearly [7]. Projections based on current smoking prevalence rates predict that, among persons who are currently younger than 18, approximately 6.4 million will die prematurely from a tobacco-related illness [7]. Financially, smoking costs approximately \$157 billion in annual health-related economic losses [6].

This chapter is intended to provide a state-of-the-art review of the pharmacology of nicotine to lay the foundation for furthering the understanding of nicotine’s role in tobacco use and its potential use as a treatment for tobacco use disorders. Research in recent decades involving the understanding, prevention, and treatment of tobacco dependence has already led to breakthroughs that are saving lives, but much has yet to be done. Based on the current course, if stronger actions are not developed and implemented, more than 1 million people will die prematurely every 2.5 years in the United States for decades to come, and approximately one-half billion of current smokers will perish globally. As recognized by the WHO, in its treaty, research is vital to guide public health policy [5]. We begin the chapter with a review of nicotine pharmacology (Section 15.2), followed by a review of pharmacologically based treatments for tobacco dependence (Section 15.3), and conclude with a section on public health policy implications.

15.2 PHARMACOLOGY OF NICOTINE

15.2.1 Overview

This section focuses on nicotine pharmacology (Section 15.2.2), namely nicotinic acetylcholine receptors (nAChRs), the neurobiological mechanisms and/or functional adaptations associated with nicotine reinforcement (Section 15.2.3),

dependence and tolerance (Section 15.2.4), withdrawal (Section 15.2.5), and nicotine pharmacokinetics (Section 15.2.6). The content (e.g., selection and order of topics) presented in Sections 15.2.2–15.2.5 has been adapted from Markou, Koob, and Henningfield (2003) [8].

15.2.2 Nicotinic Acetylcholine Receptors

Nicotine was first isolated from the leaves of tobacco, *Nicotiana tabacum*, in 1828 by Posselt and Reiman. It is a natural liquid alkaloid structurally similar to acetylcholine (ACh) that interacts with specific nAChRs in the central and peripheral nervous systems [9–11]. Nicotine and other nicotinic acting agents were used by Langely and colleagues in the 1890s and early twentieth century to explore the function and structure of the nervous system. They employed pharmacological tools, including the study of dose–response relationships, temporal and dose-related aspects of tolerance development and loss, and interactions among agonists and antagonists. This work led Langley (1905) to postulate the existence of some “receptive substance” which mediated the effects of nicotine and other agents and which would explain the selective action of differing chemicals on the same muscular and organ systems.

It is now known that nicotine produces its varied effects by binding to nAChRs, of which there are two categories, muscular and neuronal [12]. These receptors vary in their expression and function. For instance, muscular nAChRs are expressed in mature skeletal neuromuscular junctions while neuronal nAChRs are expressed in the autonomic ganglia and the central nervous system (CNS) [12, 13]. In the context of nicotine addiction and its neurobiological mechanisms, the nAChRs of most relevance are those expressed in the brain, attributable to their proposed role in the neuromodulation of several CNS transmitters [14], as described below.

Nicotinic acetylcholine receptors in the brain, located primarily on presynaptic terminals [14] and to a lesser extent at somatodendritic, axonal, and postsynaptic locations (for review, see [15]), are diverse members of the neurotransmitter-gated ion channel superfamily [16] comprised of five nAChR subunits (i.e., α and β subunits combined or α subunits alone) that combine to form a functional receptor [8, 16–18]. The nAChRs expressed most widely in the brain are comprised of α_4 , β_2 , or α_7 subunits [11, 17]; nicotine-induced dopamine release is dependent on the β_2 subunit [19]. Importantly, the way in which individual subunits (α_1 to α_{10} ; β_1 to β_4 [11, 18, 20]) combine to form a nAChR influences its affinity for nicotine binding [11, 16]. For instance, nAChRs formed in a heteropentameric configuration comprised of α and β subunits, which contain β_2 subunits, have a high affinity while nAChRs formed in a homopentameric configuration comprised only of α_7 subunits have a low affinity [16]; among nAChR subunits, β_2 has the highest affinity for nicotine [21].

Activation of nAChRs in the brain releases multiple neurotransmitters, including dopamine (DA), serotonin (5-HT), glutamate (Glu), γ -aminobutyric acid (GABA), and endogenous opioid peptides (EOPs) [16]. These neurotransmitters are associated with nicotine’s reinforcement, dependence and tolerance, and withdrawal, as described below.

15.2.3 Nicotine Reinforcement Neurosubstrates

In the context of substance abuse, nicotine has been known for decades to be dependence producing [22]; nicotine dependence diagnostic criteria have been defined

elsewhere [2]. Nicotine's dependence-producing properties are, in part, due to its pharmacodynamic profile. Nicotine serves as an effective reinforcer in humans and in several species of animals, including rats, mice, and nonhuman primates, as demonstrated by self-administration studies [23, 24]. In humans, nicotine's reinforcing effects include mild euphoria [25], increased energy and arousal, and decreased stress and anxiety and appetite [26]. Recent studies of the trajectory of patterns of acquisition of nicotine self-administration in rats may serve as a bridge to human longitudinal work. For instance, studies by Lanza et al. [27] and Donny et al. [28] demonstrate that individual differences appearing early in the acquisition process affect resulting patterns of nicotine self-administration, which is consistent with findings that individual differences in early tobacco use may influence differentially the development of regular tobacco use and the emergence of dependence in humans [29].

Neurosubstrates involved in nicotine reinforcement include the mesolimbic dopaminergic system (for reviews, see [11, 30]) and the following neurotransmitters: DA, Glu, and GABA [8]. Other neurosubstrates involved include corticotropin-releasing factor (CRF) and opioid receptors [8]. The role(s) of each neurosubstrate in the modulation of nicotine reinforcement has been provided below.

Acute nicotine administration, possibly by activating nAChRs on mesolimbic dopaminergic neurons at the ventral tegmental area (VTA) and nucleus accumbens [31], increases the firing rate of VTA dopaminergic neurons [32] and elevates dialysate DA levels specifically in the nucleus accumbens shell [33–36]. A series of studies by Nisell et al. [36–38] have demonstrated that nAChRs in the VTA, compared to those in the nucleus accumbens, have a more salient role in mediating the effects of nicotine on DA release. Further evidence of the role of nAChRs on VTA dopaminergic neurons in nicotine reinforcement has been provided by findings that injections of dihydro- β -erythroidine, a nicotinic antagonist, into the VTA [39], microfusion of 6-hydroxydopamine into the nucleus accumbens, producing mesolimbic dopamine system lesions [40], and administration of DA receptor antagonists (SCH23390 and spiperone) [41] decrease nicotine self-administration. These findings are specific to nicotine's reinforcing effects, as opposed to other potential nicotine-induced effects (cognitive improvement) [42].

Another neurosubstrate that may mediate nicotine reinforcement is the Glu neurotransmitter system [8]. Nicotine may elevate striatal DA levels either directly, by glutamatergic stimulation of the ventral striatum, and/or indirectly, by glutamatergic stimulation of VTA dopaminergic neurons projecting to the striatum [8]. By activating excitatory presynaptic nAChRs on glutamatergic terminals, nicotine increases glutamate release [43, 44]. As in other brain sites, acute nicotine administration increases Glu release in the VTA [45]. Within the VTA, nicotine is thought to act at presynaptic α_7 nAChRs located on Glu afferents [45], whereby increasing Glu release, which in turn stimulates DA release in the nucleus accumbens [45–47]; multiple studies suggest α_7 nAChR subunits and/or $\alpha_4\beta_2$ nAChR subtypes have a role in nicotine reinforcement, DA release, and the anxiolytic effects [19–50] that contribute to continued tobacco use [22] (alternatively, see [51]). As an intermediary step, the enhanced Glu release acts at *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor sites on postsynaptic dopaminergic neurons, which increases their firing rate [8]. Blockade of metabotropic glutamate receptor 5, using the mGluR5 antagonist 2-methyl-6(phenylethynyl)-pyridine, decreases nicotine self-administration in rats and mice, potentially by decreasing nicotine-stimulated DA release in the mesolimbic system [52].

Another neurosubstrate that may mediate nicotine reinforcement is the GABA neurotransmitter system [8]. Dopaminergic neurons projecting from the VTA to the nucleus accumbens [53] receive descending GABAergic input from the ventral pallidum and the nucleus accumbens [54, 55]. Dopaminergic tone in the VTA and nucleus accumbens is inhibited by these GABAergic neurons [11, 56]. At the VTA, DA inhibition involves GABAergic inhibitory afferents to dopaminergic ventral tegmental neurons [54, 57] and interneurons within the VTA [58]. In the nucleus accumbens, DA inhibition involves medium spiny GABA neurons [58]. Demonstrative of GABA's neuromodulation effects, administration of γ -vinyl-GABA (GVG, or vigabatrin), an irreversible GABA transaminase inhibitor [59], abolishes expression and acquisition of conditioned place preference, nicotine-induced increases in synaptic DA [60], and dose dependently decreases nicotine self-administration in rats [59]. Additionally, administration of baclofen or CGP44532, selective GABA_B agonists, decreases nicotine self-administration [61–63], suggesting that enhancement of GABA transmission via GABA_B receptors may antagonize nicotine's reinforcing effects. Baclofen's effects on tobacco smoking has been examined in only one clinical study [64]. Acute administration of baclofen was not demonstrably efficacious in reducing cigarette smoking or nicotine-craving ratings.

Other neurosubstrates that may mediate nicotine reinforcement involve the hypothalamic–pituitary–adrenal (HPA) axis and corticotropin-releasing factor [8] (CRF; also abbreviated CRH, for corticotropin-releasing hormone [65]), a neuropeptide neurotransmitter involved in stress responses [65]. Corticotropin-releasing factor transmission in the paraventricular nucleus of the hypothalamus (PVN) has been hypothesized to mediate the effects of acute nicotine exposure on the HPA in rodents and humans [8]. In rats, injection of nicotine or exposure to cigarette smoke increases corticosterone and adrenocorticotrophic hormone (ACTH) levels [66, 67]. A series of studies by Matta et al. (1987) [68] suggest these effects are mediated by central nicotinic cholinergic receptors. Several findings indicate that CRF in the paraventricular nucleus mediates the effects of nicotine on the HPA stress response system. First, nicotine stimulates CRF release in vitro from the rat hypothalamus [69]. Second, nicotine produces concentration- and time-dependent increases in CRF messenger RNA expression in the AR-5 immortalized amygdalar cell line [70]. Third, CRF-containing synaptic vesicles are located in axon terminals with nAChRs in the rat hypothalamus (i.e., median eminence), indicating that nicotine may act on nAChRs in axon terminals to release CRF [71]. Lastly, nicotine administration induces dose-dependent cFOS expression in CRH-containing regions of the PVN [72], bed nucleus of the stria terminalis and central nucleus of the amygdala, and dorsal raphe [73].

In humans, cigarette smoking or nicotine infusions (i.e., intravenous administration), under limited conditions, increases cortisol and ACTH levels [74–78]. One study [77] demonstrates that within a 10-min smoking period smoking two conventional (2-mg nicotine) cigarettes compared to two very low nicotine (0.2-mg nicotine) cigarettes increases cortisol levels. Another study [78], using controlled smoking procedures (e.g., interpuff interval of 25 s), demonstrates that within a 12-min smoking period 24 puffs (equivalent of two cigarettes, approximately) of high-nicotine (15.48 mg nicotine; [79]) cigarettes, but not low-nicotine (1.1-mg nicotine, as determined by the manufacturer, Murty Pharmaceuticals Inc.) cigarettes, increases ACTH levels. A third study [75], having similar design features as those above,

reveals a comparable pattern of results for both cortisol and ACTH levels. Like findings have been reported elsewhere [80]. In nonsmoking, nicotine-naïve subjects receiving intravenous nicotine bitartrate (0.25 and 0.5 µg/kg/min), only infusions of the 0.5-µg dose increase cortisol and ACTH levels [76].

Overall, the relationship between the HPA axis/hormones and abuse-related effects of drugs is not understood fully [78]. However, HPA axis activation is believed to be involved in several phases of the addiction process [78]. Multiple hypotheses regarding the involvement of the HPA axis/hormones have been raised. One hypothesis, using cocaine as a comparator, posits that ACTH and cortisol may contribute to nicotine's reinforcing effects [78]. Another hypothesis proposes that CRH may play a role in mediating nicotine's effects on behaviors pertaining to stress and anxiety [8], generally, and the anxiety and irritability often associated with nicotine withdrawal, specifically [11, 78], as described below (Section 15.2.5).

The final neurosubstrate presented in this chapter that may mediate nicotine reinforcement involves opioid receptors. Two primary lines of evidence suggest opioid receptors at least partially mediate nicotine reinforcement. First, nicotine influences the release of EOPs [81, 82], both within and outside the mesolimbic DA system [11]. Within the mesolimbic DA system, nicotine increases tissue levels of opioid peptides in the nucleus accumbens [83, 84], which contain a high density of µ-opioid receptors [85]. These receptors have been hypothesized to be occupied by endogenous opioid ligands released by nicotine [86]. Outside the mesolimbic DA system, nicotine induces the release of the pro-opiomelanocortin peptide group by stimulating nAChRs within the hypothalamus; the pro-opiomelanocortin peptide group includes the precursor β-endorphin [11, 87] which, while undetermined, may be involved in mediating nicotine's positive reinforcing effects [11]. Taken together, nicotine's positive reinforcing effects may be mediated by activation of enkephalin neurons (i.e., dopamine-independent reward systems) [11]. Second, opioid receptor antagonists such as naloxone and naltrexone have demonstrable efficacy in decreasing cigarette consumption and self-reported smoking satisfaction and increasing smoking cessation rates [88–91], suggesting that opioid receptors may modulate nicotine's reinforcing effects [8] (alternatively see [92, 93]).

15.2.4 nAChR Functional Adaptations

Features common among drugs of abuse are their ability to produce and maintain dependence and tolerance [2]. For nicotine, both dependence and tolerance may involve functional adaptations of nAChRs [8, 11, 18, 94], as described below. Contrary to most agonists, which downregulate receptors with chronic drug exposure [94], chronic nicotine administration desensitizes and inactivates nAChRs, which leads paradoxically to an upregulation of nAChR sites [8, 94, 95]. This nicotine-induced, paradoxical upregulation of nAChRs has been observed in the rodent brain [96, 97], human brain [98–100], and human blood leukocytes [101] and is dose dependent [99, 101].

The role of nAChR desensitization and upregulation in the subjective effects of acute nicotine exposure and in the development and maintenance of nicotine dependence has undergone much speculation [102, 103]. Experimental findings pertaining to the effects of chronic nicotine exposure on nAChRs in animals have been mixed. For instance, there is some evidence that chronic nicotine exposure

increases nAChR numbers [104, 105] and function [106]. Conversely, there is some evidence that chronic nicotine exposure decreases nAChR numbers [107] and function [108]. Behaviorally, as a means of counteracting the continuous agonist actions of nicotine on the receptors, nicotine dependence is related to the decrease in nAChR numbers and/or function [8, 109]. In turn, the prolonged functional desensitization or inactivation of nAChRs associated with chronic nicotine administration has been hypothesized to lead to receptor upregulation [107, 110, 111] (alternatively, see [112]). Importantly, because the majority of studies indicating nAChR changes have been conducted *in vitro*, the functional significance of these changes *in vivo* is unknown [103] and thus requires further study [113]. Also worth noting, particularly when interpreting seemingly opposite effects, is that different nAChR subtypes vary in their sensitivity to nicotine, as evidenced by differential degrees and rates of desensitization and upregulation [8, 11]. For instance, nAChRs composed of $\alpha_4\beta_2$ subunits desensitize slowly [18] while α_7 receptors desensitize rapidly [18]. Thus behavioral observations may reflect the combined effects of complex adaptations of different nAChR types [8].

It appears plausible that initial discomfort associated with smoking cessation reflects the 300 to 400% increase in nicotine receptors [100], many of which are abruptly unoccupied. In turn, nicotine replacement therapy (NRT) may provide its benefits, in part, by occupying these receptors, thus contributing to stable physiological functioning while the person behaviorally adapts to living without smoking. The therapy, in turn, provides a means to reduce gradually daily nicotine over several weeks or months, potentially, allowing receptor levels to achieve an appropriate balance. The question of returning to “baseline” or “normal” receptor levels may not be meaningful for tobacco users because they typically began smoking during adolescence with long term cessation achieved (if it is achieved) after several decades of tobacco use. The possibility that some tobacco users will require long-term NRT use (or other therapeutic measures) to sustain tobacco-delivered nicotine abstinence is then not surprising.

The aforementioned nAChR adaptations likely are involved in producing tolerance to some of nicotine’s acute effects [114]. Tolerance to nicotine’s acute effects is gained throughout the day, with repeated acute nicotine exposure, and lost throughout the night, due to overnight abstinence while the smoker sleeps [115]. The time course of tolerance, whether a gain or loss, varies across nicotine-induced responses [114, 116, 117]. For instance, smokers develop a large degree of tolerance to nicotine’s acute subjective and cardiovascular effects [114, 118–120].

15.2.5 Nicotine Withdrawal Neurosubstrates

As with many other drugs of abuse, nicotine, upon abrupt cessation (i.e., smoking abstinence in humans), can produce an aversive withdrawal syndrome in humans [121, 122] and rodents, though observed less reliably in mice [109, 123–127]. The nicotine withdrawal syndrome, as defined by the APA [2], consists of subjective, cognitive, and physiological components and has been cited by many smokers as a reason for failed quit attempts [128], which results in continued tobacco use. Common subjective effects include restlessness, impatience, irritability, depressed mood, dysphoria, craving, and anxiety [2, 121, 129]. A common cognitive effect includes difficulty concentrating [2, 121, 129]. Common physiological effects include

decreases in heart rate and adrenaline and noradrenaline excretion and increases in skin temperature, electroencephalographic theta power, and weight [130–133]. In rats, the most prominent signs of withdrawal include abdominal constrictions (writhes), gasps, ptosis, facial fasciculation, and eyeblinks [124, 134, 135]. These withdrawal signs are mediated both centrally and peripherally [11, 124, 136]. Other withdrawal signs observed in rodents include disruptions of food-maintained learned behaviors in rats [137], increases in the acoustic startle response in rats [138], decreases in prepulse inhibition in mice [127], and elevations in brain reward thresholds [109, 135, 139]. Several dissociations between threshold elevations and somatic signs [134, 139] suggest that the various components of nicotine withdrawal are mediated by different substrates [8]. Neurosubstrates involved in nicotine withdrawal, comprised of many of the same substrates involved in nicotine reinforcement, include the mesolimbic dopaminergic system and the following neurotransmitters: DA, 5-HT, and Glu [8]. Other neurosubstrates involved include CRF and opioid receptors [8]. The role(s) of each neurosubstrate in nicotine withdrawal has been provided below.

Precipitated nicotine withdrawal, as induced by systemic or intra-VTA mecamylamine (nAChR antagonist) administration, decreases DA dialysate levels in the nucleus accumbens [140, 141] and the central nucleus of the amygdala [142] in nicotine-treated rats. Additionally, mecamylamine injections into the VTA dose dependently precipitates a constellation of somatic withdrawal signs [143], suggesting possibly that nAChR transmission in the VTA has a role in the expression of nicotine withdrawal's somatic signs [8]. Similar decreases in DA levels in the nucleus accumbens have been observed with withdrawal from other drugs of abuse, including ethanol, morphine, cocaine, and amphetamine [144, 145]. Further demonstrative of the involvement of DA in nicotine withdrawal is the finding that bupropion (Zyban), a smoking cessation aid that acts, in part, by inhibiting neuronal reuptake of DA, enhances DA transmission [146, 147]. Moreover, while its effects on nicotine self-administration have been varied [148–150], bupropion has been shown to reverse threshold elevations and somatic signs associated with nicotine withdrawal [135, 151].

Because administration of nAChR antagonists into the VTA decreases nucleus accumbens dialysate levels, as described above, suggests that reductions in endogenous cholinergic tone may cause nicotine withdrawal. The receptors involved most likely are α_4 -containing high-affinity nAChRs [152].

Another neurosubstrate that may mediate nicotine withdrawal involves the 5-HT neurotransmitter, generally, and the 5-HT_{1A} receptor, specifically [153, 154]. Pre-treatment with 8-OH-DPAT and LY274600, 5-HT_{1A} receptor agonists, enhance the auditory startle response observed during nicotine withdrawal, whereas NAN-190, WAY-100635, or LY206130, 5-HT_{1A} receptor antagonists, blocks the increase in startle response [155]. Additionally, during nicotine withdrawal, the sensitivity of dorsal raphe nucleus neurons to 8-OH-DPAT increases [156]. Therefore, nicotine withdrawal, by increasing potentially the inhibitory influence of somatodendritic 5-HT_{1A} autoreceptors located within the raphe nuclei, may decrease 5-HT release into forebrain and limbic brain sites [157, 158]. Evidence of this is provided by the finding that, in rats undergoing nicotine withdrawal, coadministration of fluoxetine, a serotonin-selective reuptake inhibitor, and p-MPPI, a 5-HT_{1A} receptor antagonist, reverses elevation of brain reward thresholds but not somatic signs [139], lending further support that nicotine withdrawal's affective and somatic components are not

mediated by the same mechanism [8]. Consistent with the overall findings above, buspirone, a partial 5-HT_{1A} agonist, demonstrates efficacy in smoking cessation trials and may alleviate withdrawal severity, at least among short-term symptoms, in abstinent smokers [159–161] (alternatively, see [162]).

Another neurosubstrate that may mediate nicotine withdrawal [8], particularly in light of its role in nicotine reinforcement (Section 15.2.3), is the Glu neurotransmitter system. Because Glu neurotransmission stimulates DA release [45, 47], there is reason to believe that decreases in Glu transmission may have a role in mediating nicotine withdrawal. Contrary to expectation, this hypothesis is not supported empirically. For instance, LY354740, a Glu analog with agonist activity at Group II metabotropic glutamate receptors (mGluRs), dose dependently attenuates enhanced auditory startle responding in rats undergoing nicotine withdrawal [138], suggesting that enhanced Glu transmission is involved, at least with this aspect of nicotine withdrawal [8]. Further investigation in different brain sites is necessary to determine the role of Glu in nicotine withdrawal [8].

Other neurosubstrates that may mediate nicotine withdrawal involve the CRF system and the HPA axis [8]. Cigarette smoking elevates salivary cortisol levels in habitual smokers [163]. During short-term tobacco abstinence, cortisol levels remain virtually unchanged [164]. However, during long-term tobacco abstinence, cortisol levels decrease [165, 166]. There is rapid tolerance to nicotine's effects on plasma corticosterone [158]. The effects of nicotine withdrawal on corticosterone levels are mixed. There is evidence that nicotine withdrawal increases, decreases, or has no effect on corticosterone levels [157, 167, 168]. Further study is necessary to characterize more fully the effects of nicotine withdrawal on CRF transmission and HPA axis function [8].

The final neurosubstrate presented in this chapter that may mediate nicotine withdrawal involves opioid receptors [8]. There is evidence that naloxone, an opiate antagonist, precipitates an abstinence syndrome in nicotine-dependent rats [169]. In a related experiment, morphine, an opioid receptor agonist, reverses signs of spontaneous nicotine withdrawal [169]. Another study demonstrates that nicotine attenuates naloxone-induced jumping behavior in morphine-dependent rats [170]. Taken together, these findings suggest that common neurobiological substrates may mediate nicotine and opiate withdrawal. Findings from another study [134] reveal that, while only high naloxone doses precipitate threshold elevations and somatic signs in nicotine-treated rats, comparably lower doses induce conditioned place aversions. These results indicate that, while brain reward thresholds and somatic withdrawal signs may not be sensitive to alterations in opioid transmission, conditioned motivational states may be [8]. Moreover, findings that naltrexone and naloxone antagonize nAChRs suggest that opioid receptor antagonists may precipitate nicotine withdrawal [171], at least in part, by blocking nAChRs directly [8].

15.2.6 Pharmacokinetics

Pharmacokinetics concerns the absorption, distribution, and metabolism and elimination of a drug. This section addresses each of these areas as it pertains to nicotine, covering a range of nicotine-delivering tobacco products, as described below.

15.2.6.1 Absorption. When delivered by tobacco smoke inhalation, nicotine is carried on tar droplets as part of a complex aerosol that is inhaled into the lung. There, particles are deposited in small airways and alveoli, where nicotine is rapidly absorbed. Venous blood concentrations of nicotine rise rapidly during cigarette smoking and peak at its completion [172]. During smoking, levels of nicotine in arterial blood can more than double the levels observed in venous blood [173]. Presumably, the rapid absorption of nicotine from cigarette smoke through the lung is the result of the huge surface area of the alveoli and the dissolution of nicotine into fluid of physiological pH, which facilitates transfer across cell membranes [174].

Nicotine from chewing tobacco, snuff, and oral nicotine replacement medications (i.e., gum, lozenge, and inhaler) is absorbed through the oral mucosa. The absorption of nicotine across biological membranes depends upon the pH. The pK_a of nicotine is about 8, meaning that 50% of the nicotine is absorbed in a solution with a pH of 8. The pH of smoke from flue-cured tobaccos found in most cigarettes is acidic; thus very little of the nicotine from tobacco smoke is absorbed in the mouth. In contrast, chewing tobacco and snuff are buffered to an alkaline pH to facilitate the absorption of nicotine through the mucosal membranes [175]. Venous plasma nicotine concentrations rise fairly rapidly and reach concentrations comparable to those seen during cigarette smoking or even higher, peaking about 10–20 min after the product is removed from the mouth [176].

The pH of smoke from large cigars and pipes is somewhat alkaline; thus nicotine in the smoke of these products can be absorbed through the oral mucosa or the lung [177]. In addition, the whole tobacco aqueous pH in large cigars is alkaline, suggesting that nicotine can be absorbed into the oral mucosa directly from the cigar when the cigar is held in the mouth [177]. In contrast, the pH of the whole tobacco aqueous pH of small cigars is acidic, like a cigarette [177]. Thus, little nicotine from small cigars would be absorbed through the oral mucosa; however, like a cigarette, nicotine would readily be absorbed into the lung if the smoker inhales.

15.2.6.2 Distribution. After absorption into the blood, nicotine is distributed extensively to body tissues with a steady-state volume of distribution averaging 180 L. Based upon animal research at steady-state nicotine concentrations, spleen, liver, lungs, and brain have a high affinity for nicotine, whereas the affinity of adipose tissue is relatively low [119]. After rapid intravenous injection, concentrations of nicotine decline rapidly because of tissue uptake of the drug. Shortly after injection, concentrations in arterial blood, lung, and brain are high, while concentrations in muscle and adipose tissues are low [119]. Uptake into the brain occurs within 1–2 min, and blood levels fall because of peripheral tissue uptake for 20 or 30 min after administration. Thereafter, blood concentrations decline more slowly, as determined by rates of elimination and rates of distribution out of storage tissues [119].

Nicotine inhaled in tobacco smoke enters the brain almost as quickly as after rapid intravenous injection. Because of the delivery into the lung, peak nicotine levels in the brain may be higher, and the lag time between smoking and entry into the brain shorter than after intravenous injection [119]. The distribution half-life, which describes the movement of nicotine from the blood and other rapidly perfused tissues, such as the brain and other body tissues, is about 9 min [178], whereas the elimination half-life of nicotine is about 2 h. Distribution kinetics determine the time course of CNS actions of nicotine after smoking a single cigarette.

15.2.6.3 Metabolism and Elimination. Nicotine is rapidly and extensively metabolized, primarily in the liver but also to a small extent in the lung [119]. Nicotine's primary metabolites are cotinine and nicotine-*N*-oxide, neither of which appears to be pharmacologically active [174]. The half-life of nicotine averages about 2 h, although there is considerable variability, generally ranging from 1 to 4 h [179]. The half-life of cotinine is much longer (16–20 h), making it a much better marker of nicotine intake than nicotine itself [180]. There is evidence that mentholated cigarette smoking significantly inhibits the metabolism of nicotine, suggesting that mentholated cigarette smoking enhances systemic nicotine exposure [181]. Inhibition of nicotine metabolism occurs both by slower oxidative metabolism to cotinine and by slower glucuronide conjugation. The level of renal excretion depends on urinary pH and urine flow and accounts for 2–35% of total elimination [182].

As reviewed by Henningfield and Benowitz [3], genetic variations can affect nicotine metabolism. Specifically, the cytochrome CYP2A6 enzyme is the liver enzyme largely responsible for the metabolism of nicotine to cotinine, and difference in the expression of this enzyme can have dramatic effects on metabolism. For example, in a study of human liver microsomes, pretreatment with coumarin, a specific and selective CYP2A6 substrate, competitively inhibited cotinine formation by 85% [183]. Genetic variations that alter nicotine metabolism can affect smoking behavior. Tyndale and Sellers [184] found that there was an underrepresentation of individuals carrying defective *CYP2A6* alleles in a tobacco-dependent population and that, among smokers, those with deficient nicotine metabolism smoked fewer cigarettes. Further, inhibition of the CYP2A6 enzyme increases nicotine bioavailability by decreasing first-pass metabolism and decreases smoking [185].

15.3 PHARMACOLOGICAL TREATMENT OF NICOTINE DEPENDENCE AND WITHDRAWAL

15.3.1 Overview

Knowing that cigarette smoking has a pathophysiological basis, as evidenced by the role neurobiological mechanisms have in nicotine reinforcement and dependence, tolerance, and withdrawal, as discussed above, underscores the importance of understanding better nicotine pharmacology and the pharmacotherapies used in the treatment of nicotine dependence (i.e., known vernacularly as nicotine addiction), the foci of the chapter.

As discussed elsewhere, two distinct but typically interrelated disorders comprise what is commonly referred to as nicotine addiction: nicotine dependence and nicotine withdrawal [186]. Nicotine dependence refers to the chronic, relapsing pattern of tobacco use driven strongly by nicotine administration, whereas nicotine withdrawal is the syndrome of signs and symptoms that begin to emerge within a few hours of discontinuation of nicotine intake. Diagnostic criteria for each are provided by the APA [2] and WHO [1]. The most widely recognized goal of therapy is to help the tobacco user achieve and sustain abstinence by treatment of dependence and withdrawal [187–189]. In principle, other goals, such as temporary reduction of withdrawal symptoms to sustain temporary abstinence (e.g., in smoke-free settings) and partial nicotine replacement to enable sustained smoking reduction (i.e., “harm reduction”), are viable and have been discussed elsewhere more recently [190, 191].

As noted in Section 15.2, there are a number of neural mechanisms by which a medication may alleviate withdrawal symptoms, simulate some of nicotine's reinforcing effects, or block nicotine's reinforcing effects. These have led to the postulation of a variety of potential medications for treating tobacco dependence and/or withdrawal, as have been discussed elsewhere [187, 192]. However, the most effective and widely used medications are nicotine replacement medications and other medications that alter the reinforcing effects of nicotine.

As reviewed in Section 15.2, many of the effects of nicotine in the brain are likely to be mediated through neuromodulation in which nicotine potentiates the release of neurotransmitters, including DA, Glu, GABA, and 5-HT. By selectively activating or blocking these neurotransmitters, one might be able to mimic or block some of the reinforcing effects of nicotine.

The following sections review the available pharmacological therapies for smoking cessation and discuss potential targets for the development of new medications.

15.3.2 Nicotine Replacement Medications

The most direct way to help people manage the symptoms of nicotine dependence and withdrawal is therapeutic use of nicotine replacement medications (NRTs) [193–195] to at least partially substitute for the effects of tobacco self-administration. Nicotine medications make it easier to abstain from tobacco by replacing, at least partially, the nicotine formerly obtained from tobacco, thereby providing nicotine-mediated neuropharmacological effects. These neuropharmacological effects include increased expression and reduced turnover of nicotine receptors in the brain and other parts of the body, alteration of brain electroencephalography and regional cerebral glucose metabolism, and activation of dopaminergic reinforcement systems in the brain [196]. Nicotine is the most important substance in tobacco in that it causes and sustains addiction to tobacco. However, a variety of other substances with their own direct pharmacological actions, in interaction with nicotine, and by modulation of sensory effects undoubtedly contribute to the overall addicting effects of tobacco smoke [119, 197–200]. The importance of other substances has not been fully elucidated, but their role may be one factor limiting the effectiveness of NRT.

Laboratory research has demonstrated that animals [201] and humans [202] who have been chronically exposed to nicotine or tobacco smoke will self-administer nicotine infusions. Nicotine administration has been shown to reverse the nicotine withdrawal seen upon discontinuation of chronic nicotine exposure in rats [123] and humans [203, 204].

Currently approved NRT products include the transdermal nicotine patch and several acute NRT products, including nicotine gum, lozenge, sublingual tablet, vapor inhaler, and nasal spray. The differences in the pharmacokinetic profile of these products are illustrated in Figure 15.1.

15.3.2.1 Transdermal Nicotine Patch. Nicotine patches are applied to the skin once a day and deliver nicotine through the skin at a relatively steady rate. Use of transdermal nicotine reduces the symptoms of nicotine withdrawal, including tobacco craving [187, 194]. The nicotine patch has been shown to reduce background craving compared to placebo; however, in contrast to acute NRT formulations, the

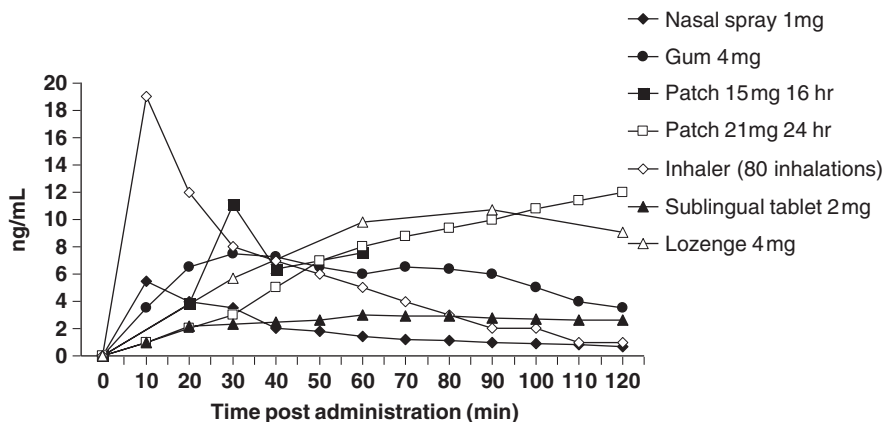


Figure 15.1 Venous blood concentrations in nanograms of nicotine per millimeter of blood as a function of time for various nicotine delivery systems. Data on nasal spray are from Schneider et al. [215]; data on gum are from Benowitz et al. [172]; data on patch are from Benowitz [204a]; data on sublingual tablet are from Molander and Lunell [212]; data on lozenge are from Choi et al. [211]; data on inhaler are from Schneider et al. [204b]; and are based on 80 puffs in 20 min, a dosing regimen not typical of clinical use.

nicotine patch has a weaker effect on craving associated with exposure to a provocative stimulus [205, 206].

Chronic nicotine also may reduce the effects of subsequent nicotine delivery, which might decrease the likelihood that a lapse will result in a complete return to smoking (relapse). For example, a study of cigarette smoking stimulant abusers indicates that nicotine patches produce tolerance to the effects of intravenous nicotine [207]. Under a placebo maintenance condition, intravenous nicotine produces robust dose-related subjective effects, with maximal increases similar to the high dose of cocaine; however, nicotine maintenance significantly decreases the subjective and reinforcing effects of intravenous nicotine. Nicotine patches do not alter responses to cocaine or caffeine.

There are currently four patch formulations on the market, varying widely in their design, pharmacokinetics, and duration of wear (i.e., 24- and 16-h wear). The diversity in patch systems has been described in reviews [194, 208], and the differences in pharmacokinetics have been illustrated in a head-to-head clinical trial [209]. All of the patch types are available in a range of dosages, and progressively lower doses are used to provide weaning over a period of several weeks or longer to enable gradual adjustment of their bodies to lower nicotine levels and ultimately to a nicotine-free state. Some formulations and indications also provide for less dependent smokers to use a lower dose.

15.3.2.2 Acute Delivery Systems. There are several options available to smokers that, unlike the nicotine patch, allow them to self-administer a dose of nicotine on an “as-needed” basis. These include nicotine gum, lozenge, sublingual tablet, oral inhaler, and nasal spray. All of these products except the nasal spray deliver nicotine through the oral mucosa. Acute-dosing products have the benefit that both the amount and timing of doses can be titrated by the user. Thus, smokers with more

nicotine tolerance or greater need can get a higher nicotine dose, and smokers who are experiencing acute adverse effects can scale back their intake.

Nicotine gum is available in two doses: 2 and 4 mg, delivering approximately 1 and 2 mg, respectively [210]. A 1-mg lozenge has been available in some European countries for some time; however, no efficacy data are available, and the efficacy of this low dose is in question. A newer nicotine lozenge, available in 2- and 4-mg formulations, has been approved in the United States, Europe, and Australia. Single-dose studies demonstrate 8–10% higher maximum concentration (C_{\max}) and 25–27% higher area-under-the-curve ($AUC_{0-\infty}$) values from lozenges compared to gums at both 2- and 4-mg dose levels, which is probably due to the residual nicotine retained in the gum [211]. A small sublingual nicotine tablet has been developed and is currently being marketed in many European countries but is not yet available in the United States. The product is designed to be held under the tongue, where the nicotine is absorbed sublingually over about 30 min. The product that is currently available contains 2 mg nicotine, and the levels of nicotine obtained by use of the 2-mg tablet and 2-mg nicotine gum are similar [212]. The nicotine vapor inhaler consists of a mouthpiece and a plastic cartridge containing nicotine. When the inhaler is “puffed,” nicotine is drawn through the mouthpiece into the mouth of the smoker. Each inhaler cartridge contains 10 mg nicotine, of which 4 mg can be delivered and 2 mg is absorbed with intensive use [213]. The product does not deliver nicotine to the bronchi or lungs, but rather its nicotine is deposited and absorbed primarily in the mouth, much like nicotine gum [214]. The nasal spray was designed to deliver doses of nicotine to the smoker more rapidly than other NRT products. The device is a multidose bottle with a pump that delivers 0.5 mg of nicotine per 50- μ L squirt. Each dose consists of two squirts, one to each nostril. Nicotine from the nasal spray is absorbed into the blood more rapidly than from gum [215].

As shown in Figure 15.1, nicotine delivery from these acute NRT formulations is faster than transdermal delivery. However, there remains a vast difference in the pharmacokinetic profiles of cigarettes and NRT products. Even nicotine nasal spray, which produces measurably faster increases in venous blood nicotine levels compared to other oral NRT formulations, does not equal the venous levels produced by cigarettes. Even more importantly, none of the currently available formulations produces the spikes in arterial blood, the blood levels that actually enter the brain. A study by Henningfield et al. (1993) demonstrates that the arterial levels achieved by smoking are much higher than levels seen in venous blood, and the nicotine may reach the brain even faster after smoking than after intravenous dosing [173]. Speed of delivery has been shown to influence nicotine’s effects in both animals and humans [173, 216].

15.3.3 Nicotinic Pharmacotherapies

15.3.3.1 Nicotinic Partial Agonists. A partial agonist is a compound that, even at high doses, does not produce the same response as a full agonist. Because there is a ceiling on the effects of a partial agonist, it is plausible that a partial nicotine agonist would have a lower risk of adverse events and have a lower abuse potential than a medication containing nicotine. A variety of nAChR subtypes have been identified with distinct structural and functional properties. The subtype that has generally been identified as being associated with the addictive effects of nicotine is the $\alpha_4\beta_2$. It is plausible that a compound that binds with a high degree of specificity or with a

greater affinity to this subtype relative to nicotine will have a higher level of safety and possibly a higher level of efficacy. However, to the extent that other subtypes might be associated with these effects, the efficacy could be muted compared to nicotine, which is less specific.

One such compound that received FDA approval in 2006 and is available as a prescription smoking cessation aid is varenicline, a nicotine partial agonist that is specific to the $\alpha_4\beta_2$ (nicotinic) receptor. Clinical trials of varenicline suggest that the medication is efficacious for smoking cessation and is safe.

15.3.3.2 Nicotinic Antagonists. In theory, a nicotinic antagonist would block the effects of cigarette smoking, which would subsequently decrease the reinforcing value of smoking, which in turn would lead to extinction of the behavior. Mecamylamine is a noncompetitive antagonist at the nAChR site. Mecamylamine increases ad libitum smoking behavior when administered alone and also attenuates smoking satisfaction as well as many of the physiological, behavioral, and reinforcing effects of nicotine [217]. There is some evidence that mecamylamine may be useful for some recalcitrant smokers as a smoking cessation aid [218]. However, the side effects of the medication (hypotension and constipation) may limit its utility.

Mecamylamine in combination with nicotine transdermal medication may produce better cessation outcomes than nicotine alone. For example, a randomized, double-blind, placebo-controlled clinical trial reveals that a combination of the nicotine patch plus mecamylamine produces abstinence rates three times higher than those for nicotine patch alone [219]. Mecamylamine also significantly reduces cigarette craving, negative affect, and appetite. Side effects such as constipation and dizziness, however, remain common. These results suggest that mecamylamine combined with nicotine replacement may ultimately prove to be a useful aid in smoking cessation.

15.3.4 Nonnicotinic Pharmacotherapies

Nonnicotine pharmacotherapies for smoking cessation have been extensively reviewed elsewhere [192, 220]. As previously mentioned, many of the effects of nicotine in the brain are likely to be mediated through neuromodulation in which nicotine potentiates the release of DA and 5-HT [221]. By selectively activating these neurotransmitters, one might be able to mimic some of the reinforcing effects of nicotine.

15.3.4.1 Bupropion. Bupropion is an atypical antidepressant drug that is the only non-nicotine-based prescription medicine approved for smoking cessation by the Food and Drug Administration (FDA). Its mechanism of action is presumed to be mediated by its capacity to block neuronal reuptake of DA and/or norepinephrine [187]. Relative to other antidepressants, bupropion has a relatively high affinity for the DA transporter [222].

Animal studies demonstrate that bupropion alters the reinforcing and withdrawal effects of nicotine. One study indicates that low doses of bupropion reduce the rewarding effects of nicotine and the affective and somatic symptoms of withdrawal [135]. Another study examined the effects of bupropion (5–40 mg/kg) on the reinforcing properties of nicotine and food in rats under two different schedules of

reinforcement [150]. The authors report that pretreatment with the highest dose of bupropion (40 mg/kg) results in a 50% reduction of nicotine intake in rats self-administering 0.03 mg/kg/infusion of nicotine under a fixed-ratio (FR) schedule. However, pretreatment with bupropion does not affect the self-administration of nicotine under a progressive-ratio (PR) schedule. These findings are challenging to interpret but may indicate that a high dose of bupropion decreases the reinforcing properties of nicotine under conditions where doses can be obtained at regular and relatively short intervals, while leaving intact the motivation to work for nicotine when doses are more widely spaced. Taken together, these results suggest that bupropion has several actions demonstrated in animals that could explain its ability to increase rates of cessation in humans.

15.3.4.2 Tricyclic Antidepressants. Tricyclic antidepressants have a relatively high affinity for both 5-HT and 5-HT transporters and some affinity for the DA transporter [222]. Several clinical trials have demonstrated the potential efficacy of nortriptyline for smoking cessation in smokers without history of major depression [223] or with such history [224], and nortriptyline has been listed by the Agency for Health Research Quality as a second-line therapy [187]. The tricyclic antidepressant doxepin has also been shown in a small human study to improve cessation rates [225]; however, larger studies are clearly needed to verify these findings. Other studies have shown that doxepin significantly reduces postcessation tobacco withdrawal symptoms and cigarette craving [226, 227].

15.3.4.3 Cannabinoid Antagonists. The cannabinoid-1 (CB1) receptor plays a role in the regulation of appetitive behavior. For example, exogenously administered cannabinoid receptor agonists stimulate food consumption in animals and humans [228]. The endocannabinoid system appears to mediate the effects of nicotine in rodents. For example, rimonabant, a CB1 receptor antagonist, appears to decrease the motivational effects of nicotine in the rat [229]. Administration of rimonabant (0.3 and 1 mg/kg) decreases nicotine self-administration (0.03 mg/kg/injection). Rimonabant (0.3–3 mg/kg) neither substitutes for nor antagonizes the nicotine cue in a nicotine discrimination procedure. In addition, using brain microdialysis, rimonabant (1–3 mg/kg) blocks nicotine-induced DA release in the shell of the nucleus accumbens and the bed nucleus of the stria terminalis. These results suggest that activation of the endogenous cannabinoid system may participate in the motivational and DA-releasing effects of nicotine.

Rimonabant is one such cannabinoid antagonist that has been recently tested in clinical trials which were reported in the news media. The study reveals that the medication was efficacious for smoking cessation. Also, smokers who quit in the rimonabant group gain less weight than those that quit in the placebo group. Many smokers report weight gain to be one of the factors associated with relapse [230]; thus a medication that reduces the weight gain associated with cessation may decrease the likelihood of relapse during a quit attempt.

15.3.4.4 Opioid Antagonists. As reviewed by Pomerleau [231], opiate agonists such as heroin or methadone have been found to increase cigarette smoking reliably in humans, and morphine has been shown to increase the potency and efficacy of nicotine in rats. There is also an extensive literature documenting the nicotine-

stimulated release of endogenous opioids in various brain regions involved in the mediation of opiate reinforcement. In addition, the opioid system may be involved in the reinforcing properties of several drugs of abuse and may be involved in nicotine's reinforcing properties. This may imply that opioid antagonists may attenuate the reinforcing value of cigarette smoking. Naltrexone is an opioid antagonist that has been shown to be effective for the treatment of alcohol dependence and has recently been approved for this indication by the FDA [232]. Two studies have examined the effects of naltrexone during smoking abstinence [92, 233]. These studies generally demonstrate little effect of naltrexone on tobacco withdrawal, smoking behavior, or satisfaction from smoking. Currently available data provide little support for the use of naltrexone for the treatment of tobacco dependence or withdrawal.

15.3.5 Nicotine Pharmacology Implications for Public Health Policy

The importance of the emerging understanding of nicotine as an addictive or dependence-producing drug over the twentieth century has had profound implications for public health policy, is the foundation for efforts by the FDA to regulate tobacco products, and was the cornerstone of the WHO Framework Convention on Tobacco Control (FCTC) [5, 234, 235]. Succinctly stated, regulatory efforts in the United States and many other countries are endeavoring to address tobacco through a combination of demand reduction and supply control efforts that recognize that tobacco use is driven and sustained critically by the pharmacological effects of nicotine.

As is evident from the preceding section of this review, many effective pharmacological treatment strategies have emerged and many more are in various stages of the drug development pipeline. The U.S. Public Health Service advocates that all cigarette smokers be offered treatment, so favorable is the benefit-to-risk ratio [187]. Globally, the WHO [189] and World Bank [236] both recommend that expanded treatment access and utilization are vital to the health, well-being, and economic development of all nations.

In the decades to come, treatment access will be vital to global health. In the very long range, however, reducing demand by more effective tobacco use prevention efforts and the prevention of escalation to dependence will be equally vital. Here, as well, nicotine pharmacology plays a potentially vital role in the needed partnership between prevention and treatment researchers. The rapid development of nicotine tolerance, dependence, and altered brain structure that contribute to persistent tobacco use are factors to be considered in public health efforts to reduce exposure to tobacco, erect barriers to reduce the conversion from any use to dependence, and develop more effective early interventions to steer the trajectory of tobacco use from addiction to abstinence. Animal and human research with nicotine and other addictive drugs has shown the importance of drug-associated stimuli in persistent use and relapse, the importance of efforts to reduce opportunities for use, and reducing overall use through increased cost. These findings are being adapted to public health policy through actions including the following: laws reducing access of youth to tobacco, increasing taxes and hence cost of tobacco products, and efforts to reduce the ubiquity of tobacco dependence-associated stimuli [5, 236, 237]. Additional approaches under consideration are parallel to those taken with medications

with abuse potential, namely, to reduce the abuse liability to the greatest degree possible without rendering the product so unacceptable that the effort would simply drive people to other forms of tobacco. Although we do not intend to over represent the importance of behavioral and pharmacological sciences in public health efforts to control one of the world's greatest public health disasters, it is clear that scientific investigation in this area has been vital to global health. As evidenced by this review, research advances have been rapid. The rapidity of these advances implies that future decades will witness even more powerful tools to bring tobacco addiction and associated disease under control. Taken together, in the decades to come, former Surgeon General C. E. Koop's vision that the end of the twenty-first century will be a time when tobacco-associated death will be as rare as it was at the end of the nineteenth century [238] eventually may be realized.

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16

PSYCHOSTIMULANTS

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16.1	Introduction	567
16.2	Neuropharmacology Related to Psychostimulant Abuse	569
16.2.1	Monoamines	570
16.2.2	Glutamate	575
16.2.3	γ -Aminobutyric Acid	576
16.2.4	Hypothalamic–Pituitary–Adrenal Axis	577
16.2.5	Neuropeptides	578
16.3	Neurobiology of Chronic Psychostimulant Exposure	579
16.3.1	Neurotransmitter and Neuroendocrine Systems	580
16.3.2	Signal Transduction Mechanisms and Gene Expression	584
16.4	Medication Development	585
16.5	Summary	588
	References	589

16.1 INTRODUCTION

Psychostimulants are a broadly defined class of drugs that stimulate the central and sympathetic peripheral nervous systems as their primary pharmacological effect. In humans, psychostimulants reliably elevate mood and promote wakefulness. In animals, psychostimulants increase locomotor activity and reliably maintain self-administration behavior indicative of robust reinforcing effects. The abuse liability of psychostimulants is well established and represents a significant public health concern. Cocaine is widely recognized as one of the most addictive and dangerous illicit drugs in use today. The most recent proceedings of the National Institute on Drug Abuse (NIDA) Community Epidemiology Work Group (CEWG), published in 2003, reported that cocaine and crack abuse was endemic in almost all 21 major U.S. metropolitan areas surveyed. Rates of emergency department visits per 100,000 population were higher for cocaine than for any other illicit drug in 17 areas, and trends in treatment admissions from 2000 to 2002 showed little change in most areas

surveyed. Drug abuse–related emergency department visits involving amphetamine or methamphetamine increased 54% in the United States between 1995 and 2002. Currently, no effective pharmacotherapy for psychostimulant abuse has demonstrated efficacy for long-term use. Clearly, a better understanding of the neuropharmacological effects of cocaine and related psychostimulants will support efforts to develop and improve useful pharmacotherapies for psychostimulant abuse.

It is important to emphasize that a number of synthetic stimulants, including amphetamines, are useful medications in the treatment of attention-deficit hyperactivity disorder (ADHD), narcolepsy, excessive daytime sleepiness, and obesity. Cocaine is still used clinically as a local anesthetic, primarily for eye, ear, nose, or throat procedures. Some examples of stimulant medications legally available in the United States and their medical indications are provided in Table 16.1. Most of these drugs are analogs of the basic phenethylamine chemical structure closely related to the catecholamine neurotransmitters norepinephrine and dopamine (Fig. 16.1). The present chapter will focus on the neuropharmacology of cocaine, amphetamine, and methamphetamine due to their high abuse potential as reflected in their categorization as Schedule II drugs (Federal Controlled Substances Act). Other stimulants have potential for abuse and dependence due to their similar profile of pharmacological effects. For example, it is well established that methylphenidate is diverted from legitimate sources, such as Ritalin, and is misused or abused by a segment of the U.S. population [1]. However, the neuropharmacology of methylphenidate will be reviewed in a separate chapter on ADHD and stimulants. Also, there are a number of illicit amphetamine derivatives, including 3,4-methylenedioxymethamphetamine (MDMA, “Ecstasy”), that have prominent stimulant and hallucinogenic properties. The latter drugs will be reviewed in Chapter 17 focused on MDMA and other “club drugs”. Recently, neurotoxicity associated with the use of amphetamine derivatives has been an area of intense investigation.

TABLE 16.1 Examples of Psychostimulants Used as Therapeutics in the United States

Drug	Trade Names	Medical Indications
Amphetamine	Adderall [®] Dexedrine [®] Dextrostat [®]	ADHD, narcolepsy, weight control
Diethylpropion	Tenuate [®]	Weight control
Methamphetamine	Adipex [®] Desoxyn [®] Methedrine [®]	ADHD, weight control
Methylphenidate	Ritalin [®]	ADHD, narcolepsy
Phentermine	Adipex-P [®] Fastin [®] Ionamin [®]	Weight control

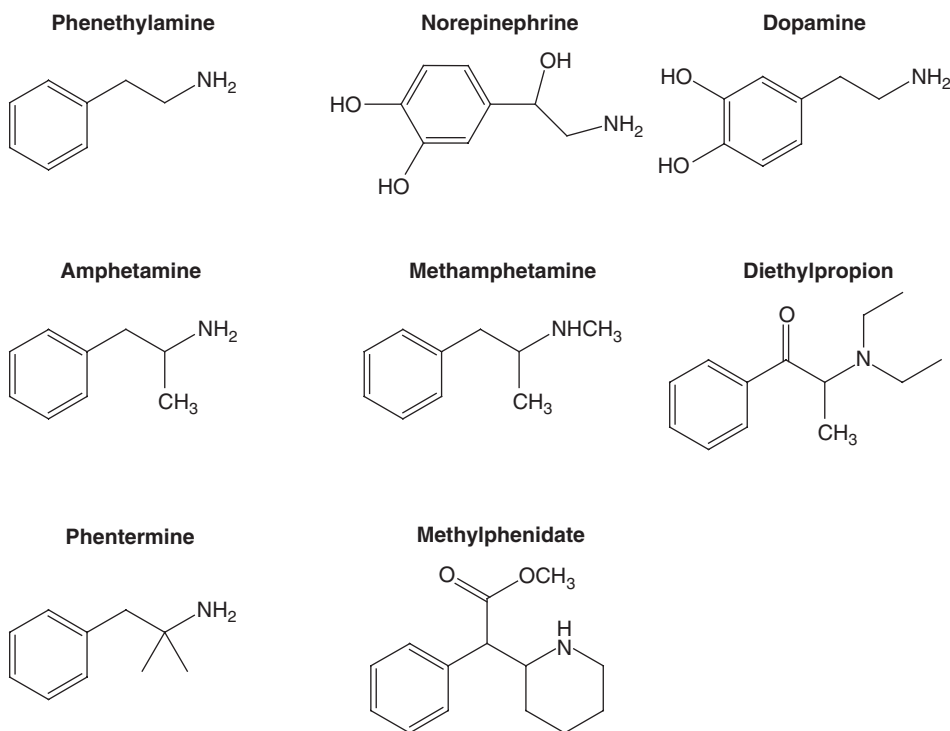


Figure 16.1 Chemical structures of endogenous and exogenous ligands for dopamine receptor. Dopamine and norepinephrine are both endogenous catecholamine neurotransmitters, sharing several chemical features, notably a catechol ring and an amine group. Exogenous ligands for the dopamine transporter, including amphetamine, methamphetamine, diethylpropion, phentermine, and methylphenidate, also share these chemical characteristics and act as psychomotor stimulants.

16.2 NEUROPHARMACOLOGY RELATED TO PSYCHOSTIMULANT ABUSE

Extensive literature documents the critical importance of monoamines (dopamine, serotonin, and norepinephrine) in the behavioral pharmacology and addictive properties of psychostimulants. In particular, dopamine plays a primary role in the reinforcing effects of psychostimulants in animals and humans. However, there is a growing body of evidence that highlights complex interactions among additional neurotransmitter, neuroendocrine, and neuropeptide systems. Cortical glutamatergic systems provide important regulation of dopamine function. Similarly, γ -aminobutyric acid (GABA) systems provide inhibitory neuromodulation of monoaminergic and glutamatergic function. Psychostimulants also activate the hypothalamic–pituitary axis and thereby engage neuroendocrine systems linked to stress reactivity. Alternatively, environmental stressors can alter the neurochemical and behavioral effects of psychostimulants. Finally, endogenous neuropeptide systems, including opioids and neurotensin, appear to play an important role in the neuropharmacology and addictive properties of psychostimulants. Drug abuse and addiction comprise a

highly complex behavioral disorder. It is not surprising that the neurobiological substrates underlying psychostimulant abuse and dependence involve a complex interplay among multiple neurochemical systems.

16.2.1 Monoamines

The primary mechanism for inactivation of monoamine signaling is transporter-mediated uptake of released monoamine neurotransmitters. Psychostimulants enhance monoamine signaling by interfering with transporter function (Fig. 16.2). However, psychostimulants differ in their relative affinity for dopamine, serotonin, and norepinephrine transporters. For example, cocaine has approximately equal

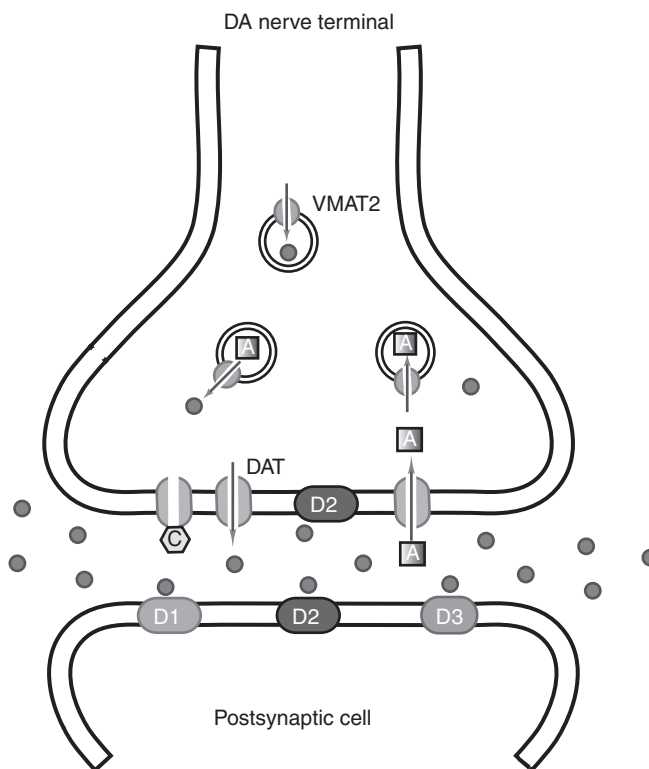


Figure 16.2 Representative dopaminergic nerve terminal and synapse. Dopamine is packaged into vesicles in the presynaptic neuron via VMAT2. Once dopamine is released into the synapse, this neurotransmitter can bind to postsynaptic dopamine receptors, including the D₁, D₂, and D₃ dopamine receptors. Dopamine D₂ receptors are also localized at the presynaptic terminal, acting as a feedback mechanism to regulate dopamine release. The DAT is also located at the presynaptic terminal and functions to remove dopamine from the synapse. Psychostimulants act at the DAT to alter normal dopamine receptor functions. Cocaine blocks the DAT, inhibiting uptake of dopamine into the presynaptic nerve terminal. Amphetamine also blocks the DAT and inhibits dopamine uptake, but also acts to release dopamine from intracellular vesicles. Abbreviations: A, amphetamine; C, cocaine; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2.

TABLE 16.2 Drug Affinities at Monoamine Transporters

Drug	Dopamine	Serotonin	Norepinephrine
Cocaine	478 ^a	304 ^a	777 ^b
(+) Amphetamine	34 ^b	3830 ^b	39 ^b
(+) Methamphetamine	114 ^c	2137 ^c	48 ^c
Methylphenidate	82 ^d	7600 ^d	440 ^d

^aIC₅₀ (nM) Matecka et al. (1996) *J. Med. Chem.* 39: 4704–4716.

^bKi (nM) Rothman et al. (2001) *Synapse* 39: 32–41.

^cKi (nM) Rothman et al. (2000) *Synapse* 35: 222–227.

^dIC₅₀ (nM) Pan et al. (1994) *Eur. J. Pharmacol.* 264: 177–182.

affinity for these three transporters (Table 16.2). In contrast, amphetamine, methamphetamine, and methylphenidate all have relatively lower affinity for serotonin transporters compared to their affinity for dopamine and norepinephrine transporters. In addition, psychostimulants differ in their actions as reuptake inhibitors versus substrate-type releasers [2, 3]. Reuptake inhibitors bind to transporter proteins and interfere with transporter function but are not transported into the nerve terminal. Cocaine is an example of a reuptake inhibitor. In contrast, substrate-type releasers bind to transporter proteins and are subsequently transported into the cytoplasm of the nerve terminal. Releasers elevate extracellular monoamine levels by reversing the process of transporter-mediated exchange, thereby enhancing monoamine efflux. They also increase cytoplasmic levels of monoamines by interfering with vesicular storage [4, 5]. Amphetamine and methamphetamine are examples of substrate-type releasers. Typically, releasers are more effective than reuptake inhibitors in increasing extracellular monoamines because the former increase the pool of neurotransmitters available for release by transporter-mediated exchange. Moreover, the effectiveness of releasers in increasing extracellular monoamines is not dependent upon the basal rate of neurotransmitter release. In contrast, the effectiveness of reuptake inhibitors is impulse dependent and, therefore, limited by the tone of presynaptic activity.

In vivo studies have demonstrated that psychostimulants can interact with multiple monoamine transporters. However, the behavioral effects of psychostimulants associated with their addictive properties have been linked most closely to enhanced dopaminergic activity. The mesocorticolimbic dopamine system comprises dopamine neurons originating in the ventral tegmental area (VTA) of the midbrain that project to several limbic and cortical structures, including the nucleus accumbens, amygdala, and prefrontal cortex [6, 7] (Fig. 16.3). Drug-induced increases in extracellular dopamine in the mesocorticolimbic dopamine system are critical in mediating the behavioral effects of psychostimulants. Two families of dopamine receptors termed D₁-like (D₁ and D₅ receptors) and D₂-like (D₂, D₃, and D₄ receptors) have been described [8, 9], and both have been implicated in the abuse-related effects of cocaine [10–12]. Evidence to support this conclusion is derived from a variety of behavioral studies characterizing the acute effects of direct-acting dopamine agonists, dopamine uptake inhibitors, and dopamine antagonists administered alone or in combination with cocaine and related psychostimulants. Operant-conditioning procedures have been implemented to characterize drug effects on behavior, including schedule-controlled behavior (model of stimulant effects),

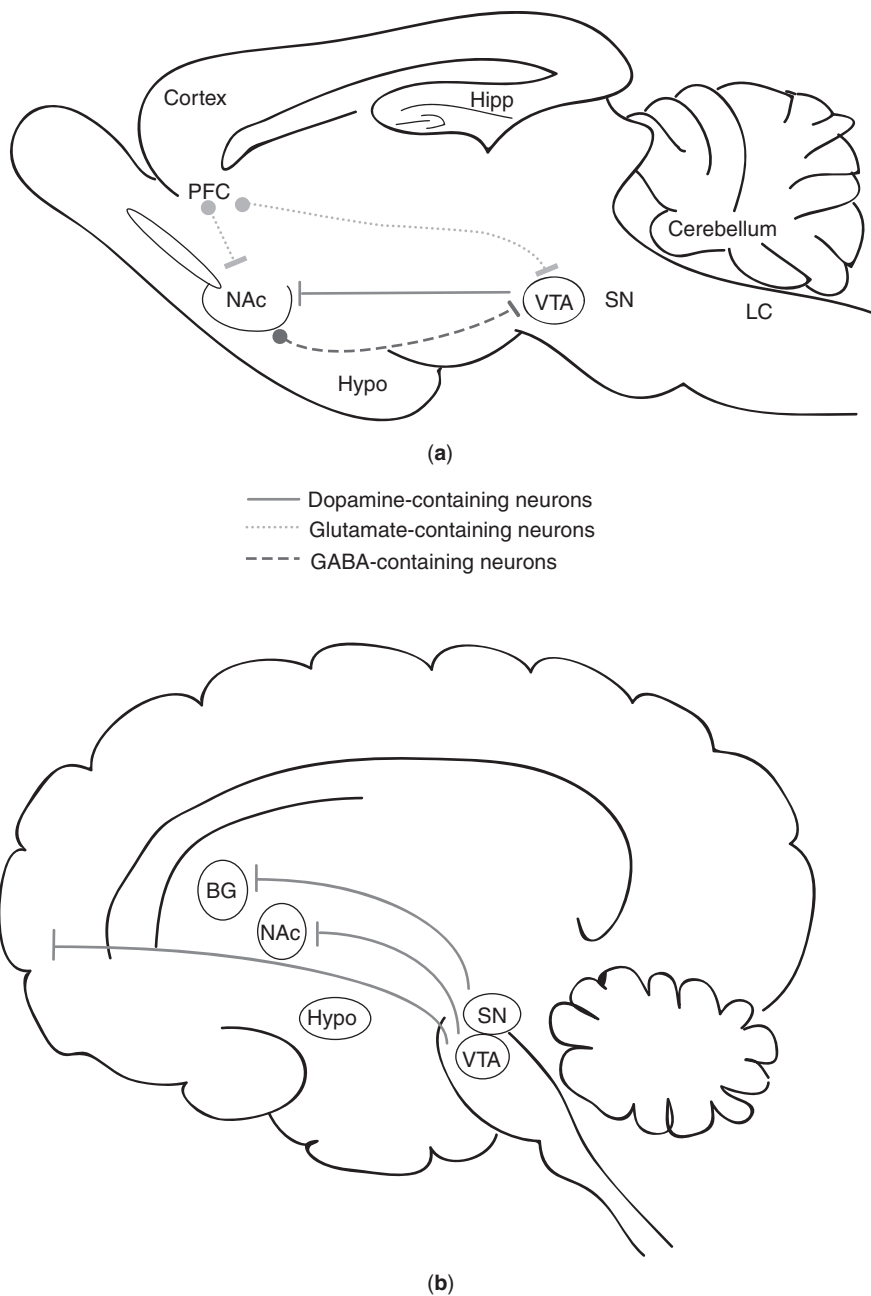


Figure 16.3 (a) Rat brain showing major components of mesolimbic system. Dopamine neurons project from the VTA and terminate in the NAc. Inhibitory GABA neurons project from the NAc and terminate in the VTA. Excitatory glutamatergic neurons project from the PFC to the NAc and to the VTA. (b) Human brain showing major dopaminergic projections. Abbreviations: BG, basal ganglia; LC, locus coeruleus; Hipp, hippocampus; Hypo, hypothalamus; NAc, nucleus accumbens; PFC, prefrontal cortex; SN, substantia nigra; VTA, ventral tegmental area.

drug self-administration (model of reinforcing effects), reinstatement (model of relapse), and drug discrimination (model of subjective effects) [13].

In a series of comprehensive studies, a significant correlation was obtained between dopamine transporter occupancy *in vitro* and the locomotor stimulant effects of cocaine analogs [14, 15]. In addition, the inhibition constants of 19 different dopamine transporter inhibitors were highly and positively correlated with their discriminative stimulus effects in rodents trained to discriminate cocaine from saline [16]. Similarly, a high correlation was found between the ability of cocaine analogs to displace [^3H]cocaine in the caudate and the ability of those compounds to produce cocaine like behavioral effects in squirrel monkeys [17–19]. Cocaine and selective dopamine uptake inhibitors exert similar effects on schedule-controlled behavior and are reliably self-administered in monkeys [17, 20–23]. Moreover, some direct-acting dopamine agonists maintain self-administration in rodents [24] and monkeys [25, 26]. Lastly, dopamine antagonists can attenuate specific behavioral effects of cocaine, including its reinforcing effects [27], its discriminative stimulus effects [28–30], and its stimulant effects on schedule-controlled behavior [20, 31–33]. The results obtained in behavioral studies provide compelling evidence that dopamine plays a major role in the neuropharmacology of cocaine.

The relevance of the dopamine transporter in the reinforcing effects of cocaine is supported further by human and nonhuman primate neuroimaging studies. In human cocaine users, a significant correlation was observed between dopamine transporter occupancy and the subjective high reported following administration of cocaine [34] or the behavioral stimulant methylphenidate [35]. Doses of cocaine within the range used by humans resulted in dopamine transporter occupancy between 67 and 69% in baboons [36]. Moreover, doses of cocaine that maintained peak response rates in drug self-administration studies resulted in dopamine transporter occupancy between 65 and 76% in rhesus monkeys [37, 38]. In addition, dopamine transporter occupancy has been determined for dopamine transporter inhibitors shown to be effective in reducing cocaine self-administration. Doses of GBR 12909 that decreased cocaine self-administration in rhesus monkeys resulted in dopamine transporter occupancy greater than 50% in baboons [39] and rhesus monkeys [38]. Similarly, doses of phenyltropane derivatives of cocaine with selectivity for the dopamine transporter decreased cocaine self-administration in rhesus monkeys at dopamine transporter occupancies between 72 and 84% [37, 38]. Collectively, these results indicate that dopamine transporter occupancy is an important determinant of the reinforcing effects of cocaine and of the effectiveness of dopamine transporter inhibitors to reduce cocaine self-administration.

The dopaminergic system is clearly an important site of action for psychostimulants, but preclinical studies have indicated that the serotonergic system can effectively modulate the behavioral effects of cocaine and amphetamine. Although compounds that selectively increase serotonin (5-HT) neurotransmission lack behavioral stimulant effects and do not reliably maintain self-administration behavior [40, 41], a negative relationship was observed between the potencies of several cocaine- and amphetamine-like drugs in self-administration studies and their binding affinities for 5-HT uptake sites [42, 43]. Coadministration of agents that induce robust increases in both dopamine and 5-HT produces minimal behavioral stimulant effects [44] and does not maintain self-administration behavior [45] in rodents. Similarly, monoamine-releasing agents have decreased reinforcing efficacy in rhesus

monkeys when 5-HT-releasing potency is increased relative to dopamine [46]. The behavioral and neurochemical profile of dopamine transporter inhibitors is also influenced by their actions at multiple monoamine transporters in squirrel monkeys [47]. Consistent with these results, administration of the 5-HT uptake inhibitor fluoxetine decreased self-administration of cocaine [48] and amphetamine [49] in rodents and self-administration of cocaine in rhesus monkeys [50]. In nonhuman primate studies, the 5-HT uptake inhibitors citalopram, fluoxetine, and alaproclate attenuated the behavioral stimulant effects of cocaine on schedule-controlled behavior [41, 51]. The 5-HT direct agonist quipazine also attenuated the behavioral stimulant effects of cocaine, whereas the 5-HT antagonists ritanserin and ketanserin enhanced the behavioral stimulant effects of cocaine [41]. Lastly, the 5-HT uptake inhibitor alaproclate attenuated cocaine self-administration and cocaine-induced increases in extracellular dopamine in squirrel monkeys [52] and cocaine-induced activation of prefrontal activity in rhesus monkeys [53]. Collectively, there is a growing body of evidence to suggest that increasing brain 5-HT activity can attenuate the behavioral stimulant and reinforcing effects of psychostimulants.

Brain 5-HT systems are ideally situated to modulate the activity of dopamine neurons and the behavioral effects of dopamine uptake inhibitors such as cocaine. Serotonin neurons from the dorsal and median raphe nuclei innervate the dopaminergic cell bodies and terminal regions of the nigrostriatal and mesolimbic dopamine systems, and the convergence of 5-HT terminals and dopamine neurons has been visualized in the VTA and nucleus accumbens at the light and electron microscopic levels [54, 55]. Serotonin can act on cell bodies to decrease the firing rate of dopamine neurons or at terminals to decrease dopamine release. In either case, the ability of 5-HT to attenuate the behavioral effects of cocaine may result from an attenuation of cocaine-induced elevation of extracellular dopamine. Alternatively, 5-HT may act postsynaptically to dopamine neurons, attenuating the effects of cocaine-induced increases of extracellular dopamine on a downstream component of the pathway.

There is a growing consensus that stimulation of 5-HT_{2C} receptors inhibits the function of the mesolimbic dopamine system [56]. Firing rates of dopamine neurons in the VTA are decreased by 5-HT uptake inhibitors [57] and selective 5-HT_{2C} agonists [58], resulting in a decrease in nucleus accumbens dopamine levels [59]. Conversely, selective 5-HT_{2C} antagonists increase the activity of these neurons [60], leading to increased dopamine release in the nucleus accumbens [61]. Since 5-HT_{2C} receptors appear to be located exclusively on GABA neurons [62], the effects of 5-HT_{2C} receptor stimulation are likely to be indirectly mediated by an enhancement of GABA-mediated inhibition of VTA dopamine neurons. Localization of 5-HT_{2C} receptors on GABAergic terminals may also explain the conflicting results which have been obtained in previous electrophysiological, neurochemical, and behavioral studies. Some studies investigating interactions between 5-HT and dopamine have concluded that 5-HT can exert an excitatory influence on dopamine activity [63] and release [64, 65]. Stimulation of 5-HT_{1B} receptors can enhance cocaine reinforcement [66], likely by decreasing GABA-mediated inhibition in the VTA [63]. The 5-HT₃ receptors also appear to play a facilitatory role in the behavioral effects of dopamine agonists [67, 68]. These seemingly disparate results likely reflect the complexity of interactions between 5-HT and dopamine systems and the 5-HT receptor subtypes influenced by drug administration.

The norepinephrine system has considerable anatomical and functional connectivity to the mesolimbic dopamine system. There is significant noradrenergic innervation of the shell subregion of the nucleus accumbens [69, 70]. The locus ceruleus, the primary norepinephrine nucleus in the brain, projects directly to the VTA and influences neuronal firing of dopamine neurons [71]. Stimulation of the locus ceruleus can increase the activity of VTA dopamine neurons, and this effect is blocked by an α_1 adrenoreceptor antagonist [72]. In addition, lesions of the locus ceruleus can decrease basal release of dopamine in the nucleus accumbens [73]. It appears that interactions between norepinephrine and dopamine may play an important role in the behavioral pharmacology of psychostimulants. For example, amphetamine-induced release of dopamine in the nucleus accumbens and conditioned place preference to amphetamine are attenuated following depletion of norepinephrine in the prefrontal cortex of rodents [74]. Lesion of the locus ceruleus or inactivation of α_1 adrenoreceptors can also attenuate amphetamine- and cocaine-induced locomotion and sensitization in rodents [75–77]. Similarly, α_2 adrenoreceptor agonists which decrease norepinephrine release via autoreceptor activation block stress-induced reinstatement of extinguished cocaine self-administration behavior in rodents [78]. Studies in nonhuman primates also support a role for norepinephrine uptake and α_1 adrenoreceptor mechanisms in the discriminative stimulus effects of cocaine [79]. There is also a significant positive correlation between potency of norepinephrine release *in vitro* and the oral stimulant dose that produces stimulant-like subjective effects in humans [80]. However, it should be noted that there is little evidence that norepinephrine plays a primary role in the reinforcing properties of psychomotor stimulants in rodents [81] or nonhuman primates [41, 82–84].

16.2.2 Glutamate

The interaction between glutamatergic and dopaminergic systems has been an area of increasing interest in drug abuse and mental health research. Anatomical substrates for glutamate–dopamine interactions have been well characterized in rodents [85]. Dopaminergic afferents from the VTA to the dorsal striatum, nucleus accumbens, and prefrontal cortex are positioned to modulate glutamate function. Conversely, the VTA, dorsal striatum, and nucleus accumbens receive significant glutamatergic innervation from a variety of brain regions, including the prefrontal cortex, hippocampus, basolateral amygdala, and thalamus. Electrophysiological studies have documented complex functional interactions between glutamatergic and dopaminergic systems that play a primary role in frontal–subcortical circuits involved in motor and cognitive function [86]. Behavioral studies have documented that interactions between dopaminergic and glutamatergic inputs in the dorsal striatum and nucleus accumbens contribute to the expression of a variety of psychomotor behaviors in rodents relevant to drug addiction [87]. Importantly, a substantial literature derived from rodent studies has documented that glutamate receptor function plays a major role in the behavioral pharmacology of cocaine and other psychomotor stimulants. In particular, glutamatergic systems have been implicated in the development of locomotor sensitization, conditioned place preference, drug self-administration, and reinstatement of extinguished drug self-administration behavior [88–92].

The excitatory effects of glutamate can be mediated by ionotropic and metabotropic receptors, and localization of both receptor families in the VTA has been documented [93]. Recent evidence indicates that metabotropic glutamate receptors (mGluRs) play an important role in the behavioral effects of cocaine associated with its abuse liability. mGluRs can be divided into three groups based on sequence homology, receptor pharmacology, and signal transduction mechanisms [94]. Group I consists of mGluR1 and mGluR5 and is linked to phospholipase C and phosphoinositide hydrolysis. Group II consists of mGluR2 and mGluR3, while group III consists of mGluR4, 6, 7, and 8. Both groups II and III are negatively coupled to adenylyl cyclase. Group II mGluRs function as autoreceptors to regulate presynaptic glutamate release [94] and as heteroreceptors to regulate release of other neurotransmitters, including dopamine [95]. Administration of an mGluR2/3 agonist decreased dopamine and glutamate release in the nucleus accumbens, striatum, and prefrontal cortex [95–98], suggesting that glutamatergic tone on mGluR2/3 suppresses extracellular levels of dopamine and glutamate. The primary origin of extrasynaptic glutamate appears to be nonvesicular glutamate regulated by cystine/glutamate transporters [98, 99]. Withdrawal from repeated exposure to cocaine in rodents led to reduced levels of extracellular glutamate in the nucleus accumbens due to reductions in cystine/glutamate exchange [100, 101]. Restoration of cystine/glutamate exchange by systemically administered *N*-acetylcysteine (NAC) normalized glutamate levels in cocaine-treated rats. Importantly, cocaine-induced reinstatement of extinguished self-administration behavior was prevented by NAC-induced restoration of extracellular glutamate. Overall, a growing body of evidence derived from rodent studies indicates that glutamate plays a fundamental role in the maintenance and reinstatement of stimulant self-administration behavior [102–104]. Lastly the mGluR5 subtype has also been implicated in cocaine self-administration. mGluR5-deficient mice did not acquire intravenous (i.v.) self-administration of cocaine, and pretreatment with the mGluR5 antagonist MPEP decreased cocaine self-administration without affecting food-maintained behavior under similar schedules of reinforcement in rats [105]. Similarly, MPEP dose dependently attenuated the development of conditioned place preference in mice [106] and suppressed cocaine self-administration behavior in squirrel monkeys [107].

16.2.3 γ -Aminobutyric Acid

The inhibitory amino acid GABA is widely distributed in the central nervous system (CNS) and can modulate basal dopamine and glutamate release [108]. The VTA contains GABAergic inhibitory interneurons that function to control the firing rate of VTA dopamine neurons [109–111]. Moreover, the majority of projection neurons in the nucleus accumbens are GABAergic neurons, some of which project to the VTA and regulate the activity of dopamine neurons [112]. Several studies have indicated that GABAergic compounds can reliably modulate the neurochemical and behavioral effects of cocaine [113, 114]. Allosteric GABA_A agonists, such as benzodiazepines and barbiturates, can inhibit dopamine and glutamate activity but have prominent sedative and hypnotic effects. However, there is considerable interest in GABA_B agents, such as baclofen, due to their attenuation of glutamate and dopamine release [108, 115, 116] and their suppression of cocaine self-administration behavior across a wide range of schedules of reinforcement and access conditions

[114, 117–119]. In addition, pharmacological inhibition of GABA transaminase, the major enzyme involved in the metabolism of GABA, can lead to a rapid increase in extracellular GABA and a corresponding attenuation of cocaine self-administration behavior at doses that do not influence locomotor activity [120, 121]. Inhibition of GABA transaminase activity with γ -vinyl GABA can also block cocaine-induced lowering of brain stimulation reward thresholds [122]. Although psychostimulants do not have direct pharmacological effects on GABAergic systems, there is convincing evidence that GABA can modulate the neurochemical and behavioral effects of psychostimulants.

16.2.4 Hypothalamic–Pituitary–Adrenal Axis

The hypothalamic–pituitary–adrenal (HPA) axis is a neuroendocrine system that responds to stress, resulting in the release of glucocorticoids from the adrenal cortex. The mature HPA axis exhibits a circadian rhythmicity with a peak around the time of waking and a trough during the quiescent time of the activity cycle. Superimposed upon this diurnal pattern is activation of stressor-specific pathways that converge in the hypothalamus, where information is integrated in the paraventricular nucleus by parvocellular neurons expressing corticotropin-releasing factor (CRF). CRF is released from nerve endings in the median eminence in response to metabolic, psychological, or physical threats and stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH, in turn, stimulates the release of glucocorticoids (cortisol in primates, corticosterone in rats) from the adrenal cortex. These steroid hormones mobilize energy substrates during stress and regulate activity of the HPA axis via negative feedback at different levels via two types of corticosteroid receptors: (a) mineralocorticoid receptors involved in the modulation of the circadian HPA rhythm and (b) glucocorticoid receptors primarily responsible for reactive negative feedback during the circadian peak and following an acute stressor. In addition to its endocrine effects, CRF has a broad extrahypothalamic distribution in the CNS [123], and its synthesis and function are affected by stress [124].

Glucocortical hormones have a facilitory role in behavioral responses to psychostimulants, including locomotor activity, self-administration, and reinstatement of extinguished self-administration behavior. These interactions appear to involve glucocorticoid effects on the mesolimbic dopamine system [125, 126]. Suppression of glucocorticoids by adrenalectomy reduces the psychomotor stimulant effects of cocaine [127] and amphetamine [128, 129], and this effect is reversed by corticosterone administration [128]. Although psychostimulants increase corticosterone secretion [130], there is no correlation between drug-induced locomotion and drug-induced corticosterone increase [131, 132]. Similarly, reductions in circulating corticosterone attenuate the reinforcing effects of psychostimulants [133, 134]. However, blocking psychostimulant-induced elevations in glucocorticoid secretion does not affect cocaine self-administration [135–138]. Finally, corticosterone appears to play an important role in cue- and stress-induced reinstatement of extinguished self-administration behavior but not drug-induced reinstatement. Ketoconazole, which decreases glucocorticoid levels, prevents reinstatement induced by drug-paired environmental stimuli [138, 139]. Similarly, basal levels of corticosterone are necessary for stress-induced reinstatement even though stress-induced increases in corticosterone are not required [140, 141]. Moreover, stress-induced reinstatement

can be blocked by corticotropin-releasing hormone (CRH) antagonists, suggesting that extra-hypothalamic CRH plays a role in stress-induced reinstatement [140, 142]. In contrast, cocaine-induced reinstatement is minimally influenced by adrenalectomy [140] and is not affected by ketoconazole administration [143].

16.2.5 Neuropeptides

All three opioid receptor subtypes (μ , κ , and δ) have been localized within dopamine systems in rodents [144–147]. Accordingly, it is not surprising to find interactions between the effects of drugs that primarily target these systems. As discussed earlier in this chapter, psychomotor stimulants interact directly with the dopamine system, either by blocking the dopamine transporter (as in the case of cocaine) or by stimulating dopamine release (as in the case of amphetamine). In contrast, opioid interactions with the dopamine systems are indirect. The μ - and δ -opioid receptors are located on GABAergic cells, which normally inhibit dopamine neurons. Activation of these receptors suppresses the activity of these inhibitory GABAergic interneurons, releasing the inhibition of dopamine cells, thereby increasing dopamine release from terminals [148–151]. In contrast, κ -opioid receptors are found on dopamine nerve terminals [152, 153], directly inhibiting dopamine release [145, 148, 154, 155].

Cocaine and heroin are often abused together in a “speedball” combination, resulting in euphoric effects that are greater than those of either drug alone [156, 157]. Preclinical research indicates that the combined administration of psychomotor stimulants and opiates enhances the effects of the individual drugs in several different behavioral assays. In rodent locomotor assays, μ -opioid receptor agonists potentiated the stimulant effects of cocaine and amphetamine [158–160]. The μ -opioid receptor agonists also potentiated the discriminative stimulus effects of cocaine in squirrel monkeys [161, 162]. However, drug self-administration studies in both rats [163] and rhesus monkeys [164] indicated that the combination of cocaine and heroin was not more reinforcing than cocaine alone. In contrast to the μ -opioid receptor agonists, the κ -opioid receptor agonist U-69593 attenuated cocaine- [165] and amphetamine- [166, 167] induced activity in rodents. Similarly, the κ -opioid receptor agonist U50488 attenuated the discriminative stimulus effects of low doses of cocaine in squirrel monkeys [168]. The results of stimulation of δ -opioid receptors on the behavioral effects of cocaine have been inconsistent [169]. The δ -opioid receptor agonist DPDPE potentiated cocaine-induced increases in locomotor activity in rodents [170]. However, the δ -opioid receptor antagonist naltrindole did not alter cocaine self-administration or cocaine-induced conditioned place preference in rodents [171]. The effects of the δ -opioid receptor agonist BW 373U86 did not alter the discriminative stimulus effects of cocaine in squirrel monkeys in one study [168], but in another study, the high-efficacy δ -opioid receptor agonist SNC80 potentiated the discriminative stimulus effects of cocaine [162].

Opiates not only influence psychomotor stimulant-induced changes in behavior but also alter psychomotor stimulant-induced changes in extracellular dopamine in the striatum and nucleus accumbens. When given in combination with cocaine, μ -opioid receptor agonists enhanced cocaine-induced increases in extracellular dopamine in rats [172, 173]. In contrast, κ -opioid receptor agonists attenuated cocaine- and amphetamine-induced increases in extracellular dopamine [167, 174].

Neurotensin is colocalized in tyrosine hydroxylase-containing dopamine neurons in the rat VTA and prefrontal cortex [175, 176]. Direct injections of neurotensin into selected brain regions results in changes in neurochemistry and behavior that are similar to those observed after administration of psychomotor stimulants. *In vitro* and *in vivo* studies show that neurotensin increases the firing of dopamine neurons in several brain regions, including the VTA [176, 177]. Intracerebroventricular administration of neurotensin increased levels of dopamine and its metabolites in several brain areas, including the striatum and nucleus accumbens [178, 179]. Infusion of neurotensin into the striatum or the VTA, but not into the nucleus accumbens, increased extracellular dopamine levels in rats [180, 181]. Repeated infusion of neurotensin into the VTA resulted in an enhanced dopamine response compared to an acute injection [180]. Locomotor activity was increased following infusion of neurotensin into the VTA of rats [180, 182] and sensitization to this behavioral effect was apparent after repeated neurotensin treatment [183]. Although rats self-administered neurotensin directly to the VTA [184], the neurotensin receptor agonist NT69L was not self-administered by rhesus monkeys [185].

Changes in dopamine neurotransmission following neurotensin administration are also reflected in alterations of psychostimulant-induced changes in neurochemistry and behavior. Direct neurotensin infusion to the striatum attenuated increases in dopamine induced by low, but not high, cocaine doses in rats [181]. In contrast, neurotensin did not block amphetamine-induced increases in dopamine in the nucleus accumbens [186]. Intravenous or intra-accumbal injections of neurotensin attenuated cocaine- and amphetamine-induced increases in locomotor activity in the rat [187–189]. Similarly, systemic administration of the neurotensin receptor agonist NT69L blocked the acute locomotor effects of cocaine and amphetamine in rats [190, 191]. Repeated, but not acute, administration of the selective neurotensin antagonist SR48692 decreased cocaine-induced increases in locomotor activity in rats [192]. Although neurotensin can alter the neurochemical and behavioral stimulant effects of psychostimulants, it may not play an important role in the reinforcing effects of these drugs. Direct application of neurotensin into the nucleus accumbens did not alter cocaine self-administration in rats [189], and the neurotensin receptor agonist NT69L did not alter the reinforcing effects of cocaine in rhesus monkeys [185].

16.3 NEUROBIOLOGY OF CHRONIC PSYCHOSTIMULANT EXPOSURE

Repeated exposure to psychostimulants can lead to robust and enduring changes in neurobiological substrates and corresponding changes in sensitivity to acute drug effects on neurochemistry and behavior. Diminished sensitivity to the effects of a drug during repeated exposure is indicative of tolerance, whereas enhanced sensitivity is indicative of sensitization. Both tolerance and sensitization have been reported to develop during repeated administration of stimulants in animal studies [193]. However, the outcome depends upon a variety of procedural variables, including the drug effect under investigation, the dosing regimen, the environmental context associated with drug administration, and the animal species. The vast majority of studies have focused on sensitization to locomotor stimulant effects in rodent models. Stimulants including cocaine and amphetamines can produce robust sensitization in

rodents, usually identified as a progressive increase in locomotor activity or stereotyped behavior with repeated drug dosing [194]. In fact, sensitization has been proposed as a general model of neural plasticity whereby drug-induced changes in behavior can be linked to concomitant changes in molecular mechanisms. It is important to emphasize that some neurobiological changes are not evident when drug administration is terminated but actually emerge during the period of drug withdrawal.

16.3.1 Neurotransmitter and Neuroendocrine Systems

There is substantial evidence that the mesocorticolimbic dopamine system and its excitatory glutamatergic inputs are critical for the development of sensitization to the behavioral effects of psychostimulants [91, 195]. Studies involving microinjection of drugs into discrete brain regions have indicated that the VTA, a region rich in dopamine cell bodies, plays a critical role in the development of sensitization. In contrast, the nucleus accumbens, a major dopamine projection area from the VTA, appears to be more closely linked to the expression of sensitization. For example, microinjections of dopamine D₁ receptor antagonists [196, 197] or glutamate *N*-methyl-D-aspartate (NMDA) receptor antagonist [198] into the VTA can disrupt the development of sensitization. However, glutamate NMDA antagonists do not block the expression of sensitization [199]. Similarly, dopamine antagonists can block the development of sensitization to psychostimulants without blocking its expression [200]. Glutamatergic afferents from the prefrontal cortex to the VTA and the nucleus accumbens have been implicated in both the development and expression of sensitization to cocaine and amphetamine [201]. Sensitized animals also reliably show an augmented response to drug-induced increases in extracellular glutamate and dopamine in the nucleus accumbens [88, 202]. Collectively, there is convincing evidence to suggest that glutamatergic afferents from the prefrontal cortex produce adaptations in the VTA that mediate the development of sensitization to psychostimulants and that secondary adaptations within the nucleus accumbens are necessary for the expression of sensitization.

Behavioral sensitization to stimulants, including cocaine, amphetamine, and methylphenidate, also involves neuroadaptation of stress-responsive systems, in particular the HPA axis and CRF pathways. In fact, there is a dynamic crosstalk between these systems. Stimulants affect HPA axis function, but there is also evidence that exposure to stress or stress hormones increases sensitivity to stimulants [136, 203]. The latter effect has been linked to glucocorticoid effects on dopaminergic neurotransmission [203, 204]. Repeated exposure to stimulants in adult rodents produces long-term increases in HPA axis activity, resulting in enhanced ACTH and corticosterone secretion [204–206]. Studies of chronic administration of methylphenidate in periadolescent rats have found similar long-term effects in adulthood, such as increased HPA axis and behavioral (anxiety-like) stress reactivity [207]. Another long-term effect of stimulants on the central components of the HPA axis of the rat includes the downregulation of hippocampal and cerebral cortex glucocorticoids that could affect glucocorticoid-induced negative feedback on HPA axis activity [208, 209]. Stimulants also induce alterations of extrahypothalamic CRF pathways [210], which could mediate the reported anxiogenic effects of chronic psychostimulant administration [204, 207, 211]. There is abundant evidence of bidirectional interactions

between sleep–wake and HPA axis activity rhythms. Sleep disturbances have been reported in adults with ADHD and include poorer sleep quality and higher nocturnal motor activity that improve after treatment with methylphenidate [212]. In healthy volunteers, stimulants seem to impair subjective ratings of sleep and increase early morning alertness [213, 214]. Although stimulants improve sleep–wake deficits in children with ADHD, the long-term consequence of chronic stimulant treatment on sleep–wake rhythms has not been established.

Sensitization to psychostimulants has been demonstrated in nonhuman primates, but studies to demonstrate sensitization in humans have yielded equivocal results. Rhesus monkeys trained to self-administer cocaine showed an augmented response to cocaine-induced elevations in striatal extracellular dopamine that emerged over a two-year period of drug exposure [215]. Chronic amphetamine exposure in nonhuman primates also induced a pattern of behavioral response that resembled the positive-like symptoms of schizophrenic humans [216]. Negative-like symptoms have also been observed in nonhuman primates following chronic amphetamine treatment [217]. More recently, a longitudinal study in rhesus monkeys exposed to repeated, escalating doses of amphetamine documented enhanced behavioral responses to subsequent acute low-dose amphetamine challenges [218]. Moreover, the enhanced behavioral responses to amphetamine challenge were evident up to 28 months postwithdrawal from chronic treatment. Several human studies in normal volunteers with no history of prior stimulant use reported evidence of sensitization to psychological (energy level and mood) and physiological (eye-blink rates) measures following two or three daily doses of amphetamine [219–221]. The outcome measures demonstrated enhanced increases following the last amphetamine dose compared to the first dose, suggesting that behavioral sensitization can be documented in human subjects. However, studies conducted in experienced stimulant users have not found evidence of sensitization. Experienced cocaine users failed to show sensitization after one or four prior cocaine exposures [222, 223]. Similarly, subjects with histories of stimulant use failed to show sensitization to oral amphetamine or methamphetamine [224, 225]. Repeated amphetamine challenges in patients with first-episode manic or schizophrenic psychosis also failed to induce sensitization [226].

There is legitimate concern that stimulant treatment during adolescence could have significant and enduring effects on reward processes relevant to mood regulation and risk for drug abuse. Preclinical studies have clearly documented that stimulants can have profound and long-lasting behavioral and neurobiological effects [91, 227]. Repeated exposure to stimulants in rodents reliably produces sensitization to their locomotor stimulant effects [228, 229] and can induce cross-sensitization with different classes of stimulant drugs [230]. Locomotor sensitization has been reported for low-dose stimulant administration intended to model therapeutic dosing [229]. Importantly, repeated dosing protocols that produce locomotor sensitization in rats can enhance the reinforcing properties of stimulants [231–234]. Once established, these behavioral and associated neurobiological changes can be remarkably stable and enduring [91, 235, 236]. Collectively, the results of laboratory studies in rodents raise significant concerns that prior exposure to stimulants, including those prescribed for the treatment of ADHD, may increase vulnerability to drug abuse in humans [237]. However, this area of investigation has received inadequate attention in human subjects and has not been approached with the experimental control and rigor afforded in animal studies. While ADHD is prevalent

in treatment-seeking substance abusers [238], clinical studies have not provided direct support for concerns that have emerged from preclinical studies. On the contrary, recent reports suggest the possibility of reduced risk for substance disorders in children with ADHD who received therapeutic administration of stimulants such as methylphenidate [239, 240]. There is an obvious need to develop clinically relevant animal models that effectively extrapolate to the human condition.

Efforts to define the long-term neurobiological consequence of psychostimulant administration have focused primarily on the dopaminergic system and have yielded inconsistent results. For example, cocaine exposure has been reported to increase, decrease, or have no effect on dopamine transporter density in rodents [241–247]. Similarly, chronic cocaine administration in rodents has been reported to increase, decrease, or have no effect on dopamine D₁ or D₂ receptor density [248–251]. The equivocal results likely reflect different dosing regimens and withdrawal periods as well as the use of noncontingent drug administration protocols that do not model voluntary drug use. Active drug self-administration protocols and periods of drug abstinence can have profound influences on neuroadaptive changes in dopamine systems [252]. Accordingly, a more consistent picture has emerged from nonhuman primate studies of cocaine self-administration. For example, in rhesus monkeys trained to self-administer cocaine intravenous for 5 days, 3.3 months, or 1.5 years, initial exposure leads to moderate decreases in dopamine transporter density in the striatum, as determined postmortem with quantitative autoradiography [253]. However, longer exposure resulted in increased striatal dopamine transporter density that was most pronounced in the ventral striatum at the level of the nucleus accumbens. Importantly, the increases in dopamine transporter binding observed after long-term cocaine self-administration in nonhuman primates correspond closely to increases observed in postmortem tissue of human cocaine addicts [254, 255]. In related studies, rhesus monkeys trained to self-administer cocaine on a daily basis over 18–22 months showed lower dopamine D₁ binding density as determined postmortem with quantitative autoradiography [256]. The effects were most pronounced in regions of the striatum where the nucleus accumbens is most fully developed. In parallel studies using the same dosing schedule and quantitative autoradiography, dopamine D₂ binding density was lower in all regions of the striatum rostral to the anterior commissure [257]. Collectively, these drug-induced changes in the status of the dopamine system may contribute to the development of dependence associated with long-term psychostimulant use.

Functional neuroimaging techniques have been used effectively in humans to characterize the long-term consequences of stimulant exposure in the context of drug abuse. Compared to controls, detoxified cocaine abusers had a marked decrease in dopamine release as measured by methylphenidate-induced decreases in striatal [¹¹C]raclopride binding [34]. The self-reports of a “high” induced by methylphenidate were also less intense in cocaine abusers. The decrease in dopamine release in the striatum has been hypothesized to underlie the decrease in sensitivity to natural reinforcers in drug abusers [258, 259]. The density of the dopamine transporter and receptors in humans has also been evaluated with positron emission tomography (PET) imaging studies. In cocaine abusers, dopamine transporter density appears to be elevated shortly after cocaine abstinence but then to normalize with long-term detoxification [260]. In contrast, PET studies characterizing dopamine D₂ receptors have reliably documented long-lasting decreases in D₂ receptor density in stimulant

abusers [261]. The reduction in D₂ receptor function coupled with dysfunctional dopamine release may further decrease sensitivity of reward circuits to stimulation by natural rewards and increase the risk for drug taking [262]. Lastly, regional brain glucose metabolism measured by FDG uptake has been characterized in conjunction with dopamine D₂ receptors [263, 264]. Reductions in striatal D₂ receptors were associated with decreased metabolic activity in the orbital frontal cortex and anterior cingulate cortex in detoxified individuals. In contrast, the orbital frontal cortex was hypermetabolic in active cocaine abusers [265]. Collectively, these findings observed in stimulant abusers document significant dysregulation of dopamine systems that are reflected in brain metabolic changes in areas involved in reward circuitry. Unfortunately, such well-designed clinical studies have not been conducted in the context of stimulant use for therapeutic purposes. However, therapeutic doses of methylphenidate block dopamine transporter function and increase extracellular dopamine [266, 267]. There is also a positive correlation between clinical improvement and reduction in dopamine transporter density in the basal ganglia following methylphenidate treatment [268]. Functional magnetic resonance imaging studies suggest that methylphenidate increases frontal cortical activity in children with ADHD [269], while PET imaging studies suggest that methylphenidate modulates brain regions associated with motor function in adults with ADHD [270]. Earlier PET studies using FDG in adults with ADHD found more limited brain metabolic effects following acute administration of *d*-amphetamine [271] and following chronic administration of *d*-amphetamine or methylphenidate [272]. Clearly, there is a need to conduct well-controlled laboratory studies to document the long-term consequences of low-dose stimulant exposure on dopaminergic function and brain metabolism.

Although significant attention has been focused on the dysregulation of the dopaminergic system, it should be emphasized that chronic exposure to stimulants can have long-term neurobiological effects on numerous neurotransmitter systems. Notably, enduring changes in glutamatergic function have been associated with repeated administration of psychostimulants. For example, basal extracellular levels of glutamate in the nucleus accumbens are decreased in rats with a history of repeated cocaine exposure [88] and there is a corresponding augmentation of cocaine-induced increases in glutamate [88, 273]. Others have reported a reduction in signaling through group I and group II metabotropic glutamate receptors [101, 274] and a reduction in sensitivity of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptors to electrical stimulation of the prefrontal cortex [275]. An enhanced inhibitory effect of dopamine on excitatory AMPA currents has also been reported [276]. The apparent downregulation of presynaptic and postsynaptic glutamate transmission following repeated cocaine exposure has been linked to cocaine-induced reinstatement of extinguished self-administration behavior and may have direct relevance toward understanding relapse to stimulant use in humans [99, 104].

There is evidence that GABAergic function is altered following chronic cocaine administration. An increase in benzodiazepine receptor binding was observed in the striatum and amygdala of rats up to 40 days after repeated cocaine administration was discontinued [277]. A sensitized behavioral response to systemically administered diazepam was also observed following the same dosing regimen [278]. These findings suggest that chronic cocaine exposure can produce alterations in GABAergic

mechanisms that may mediate aspects of drug withdrawal. Long-term alterations in the 5-HT system have also been reported. Chronic exposure to cocaine enhanced sensitivity of 5-HT_{1A} receptors to inhibit GABAergic medium spiny neurons of the striatum [279] and reduced 5-HT concentrations in the frontal cortex [280] in rats. However, a postmortem study of human cocaine users documented higher 5-HT levels in the frontal cortex compared to matched controls [281]. Dysregulation of the noradrenergic systems may also be associated with chronic cocaine exposure. Altered noradrenergic tone was observed during cocaine withdrawal in human cocaine abusers [282], and chronic cocaine self-administration in nonhuman primates upregulated the norepinephrine transporter and decreased cerebral metabolism in the bed nucleus stria terminalis, a brain region that plays a key role in cocaine withdrawal and stress-induced reinstatement of extinguished self-administration behavior [283]. Although psychostimulants do not directly interact with opiate receptors, chronic psychostimulant exposure can influence endogenous opiate systems. For example, chronic cocaine administration increased μ - and δ -opioid receptors in several rat brain regions [284]. Downregulation of κ -opioid receptors in both the nucleus accumbens and the striatum was observed after chronic administration of cocaine and amphetamine in one study [285], but no changes in κ -opioid receptors were observed after chronic administration of cocaine in another study [284]. However, postmortem brains from fatal cocaine overdose victims showed increased κ -opioid receptor binding in limbic areas [286]. Similarly, PET neuroimaging in human cocaine users showed increased μ -opioid receptor binding that correlated with self-reported cocaine craving [287].

16.3.2 Signal Transduction Mechanisms and Gene Expression

In addition to regulation of ion channels, neurotransmitters regulate virtually all progresses that occur in neurons, including gene expression. Most of the effects of neurotransmitters on target neurons are achieved through biochemical cascades of intracellular messengers. Intracellular messengers are comprised of G proteins [guanosine triphosphate (GTP) binding membrane proteins], second messengers [such as cyclic adenosine monophosphate (cAMP) and Ca^{2+}], and protein phosphoregulators [288, 289]. Monoamine receptors are coupled to G proteins which regulate adenylyl cyclase activity to modulate the second messenger cAMP. The function of cAMP is to regulate the activity of protein kinases, phospholipases, and other intracellular enzymes. In turn, the enzymes regulate a variety of intracellular processes, including gene transcription that is regulated by transcription factors. Thus, the regulation of neurotransmitter signal transduction mechanisms and changes in gene transcription and protein synthesis can alter the number and type of receptors in target neurons as well as the functional activity of intracellular systems [290]. In recent years, significant advances in molecular biology have identified important drug-induced changes in neurobiology that may play a fundamental role in the transition to addiction.

The most extensively characterized signal transduction pathways activated by psychostimulants are those associated with dopamine receptor activation. The majority of research to date has focused on the VTA and nucleus accumbens [291]. Chronic administration of cocaine upregulates the cAMP pathway in the nucleus accumbens [292]. Cocaine and amphetamine also upregulate the transcription factor

cAMP response element binding (CREB) protein in the nucleus accumbens [293, 294]. Convincing evidence suggests that these neuroadaptations can decrease sensitivity to the rewarding effects of psychostimulants and may impair the general reward system resulting in an amotivational, depressed-like state [293, 295, 296].

Acute administration of psychostimulants rapidly activates several immediate early genes in the rat brain, including *c-fos* and *c-jun* [236, 297]. The protein products of these genes function as nuclear transcription factors that regulate gene expression. Chronic exposure to stimulant can lead to tolerance of the gene activation effect and a downregulation of gene products. For example, chronic administration of cocaine significantly reduced the activity of subsequent drug exposure to induce *c-fos* and other *fos* proteins in rats [298]. Moreover, there was an accumulation of *fos*-related antigens, including the transcription factor Δ FosB [299]. Importantly, enhanced Δ FosB expression in the nucleus accumbens and dorsal striatum is associated with sensitization to the locomotor and reinforcing effects of cocaine. Overexpression of Δ FosB increased sensitivity to the locomotor stimulant and rewarding effects of cocaine and increased cocaine self-administration [300, 301]. Due to the extraordinary stability of Δ FosB, it could conceivably sustain these types of behavioral changes for weeks or months after the last drug exposure. Hence, it may serve as a molecular switch to support the initiation and maintenance of drug dependence [291].

Finally, acute administration of psychostimulants in rodents rapidly induces cocaine- and amphetamine-regulated transcript (CART) peptides in brain regions associated with reward systems, including the VTA, ventral pallidum, amygdala, lateral hypothalamus, and nucleus accumbens [302, 303]. Hence, CART is anatomically positioned to regulate mesolimbic dopamine function, and there is evidence that CART may play a role in the behavioral effects of psychostimulants. For example, administration of CART into mesolimbic regions can induce behavioral effects similar to those observed for psychostimulants. Direct injection of CART into the VTA of rats produced modest increases in locomotor activity and promoted conditioned place preference [304]. Intra-VTA injection of CART also induced modest but significant elevations in extracellular dopamine [302]. However, when coadministered with cocaine or amphetamine, CART attenuated drug-induced increases in locomotor activity [305, 306]. This functional antagonism suggests that CART peptides may be considered as potential targets for medication development to treat psychostimulant abuse [302]. It is interesting to note that CART messenger RNA (mRNA) levels were increased in the VTA of cocaine overdose victims [307, 308].

16.4 MEDICATION DEVELOPMENT

There is a growing appreciation that drug addiction is a chronic relapsing disorder with a biological basis. Significant advances in the understanding of neurobiological mechanisms underlying drug abuse and dependence have guided pharmacological treatment strategies to improve clinical outcome. Considerable effort has been directed toward the development of effective medications for substance abuse disorders and has led to useful pharmacological interventions. Notably, methadone has been an effective medication and adjunct in the treatment of heroin abuse for many years [309, 310], nicotine replacement has been effective in smoking cessation [311], and naltrexone has documented efficacy in the treatment of alcoholism [312,

313]. During the past two decades, psychostimulant addiction has been a major focus of multidisciplinary research efforts, including molecular approaches, preclinical behavioral studies, and clinical trials. However, no suitable medication has been approved for the treatment of stimulant use disorders [314, 315]. It should be noted that the vast majority of clinical research has focused on cocaine rather than other psychostimulants such as amphetamines and methylphenidate. The extent to which outcomes related to cocaine addiction can be extended to other psychostimulants remains unclear [316]. There are multiple pharmacological approaches in the treatment of cocaine abuse and dependence, including (1) functional antagonist treatments which block the euphoric effects of cocaine and extinguish illicit drug use; (2) functional agonist treatments which replace some of the pharmacological effects of cocaine, thereby stabilizing neurochemistry and behavior; and (3) treatments that attenuate symptoms of cocaine toxicity or withdrawal [314, 316]. Numerous medications have been evaluated for treatment of cocaine dependence that include a wide range of pharmacological targets. Reviews of the clinical literature have reported no significant benefit from antidepressants or dopamine agonists for cocaine dependence [317, 318]. Antagonist strategies designed to block the euphoric or positive effects of psychostimulants with antipsychotic medications have included risperidone [319], flupenthixol [320], and olanzapine [321] and have yielded negative clinical outcome largely due to poor compliance and treatment retention. Several novel approaches that have shown some clinical promise include disulfiram, a well-established medication for treatment of alcoholism [322–324], and GABA_B receptor agonists [114]. A recent review also reported promising results for agonist-like stimulant medications in the treatment of cocaine and amphetamine dependence [314].

Tricyclic antidepressants are the best-characterized class of medications for the treatment of cocaine dependence. Desipramine was the first medication reported to be effective in an outpatient, controlled clinical trial. An initial meta-analysis found desipramine to be effective in reducing relapse to cocaine use [325], but subsequent clinical trials did not confirm its effectiveness [326, 327] or found it effective only for limited periods [328]. Based on pharmacological mechanisms, there is no convincing rationale for selecting desipramine over other tricyclic antidepressants [316]. Initial human laboratory studies with the selective serotonin reuptake inhibitor fluoxetine were encouraging. A four-week inpatient study in healthy volunteers found that fluoxetine significantly decreased subjective ratings of cocaine-induced positive mood effects [329]. However, controlled clinical trials with fluoxetine have not documented significant advantages over placebo [330, 331]. Similarly, clinical effectiveness has not been documented for the antidepressants bupropion [332] and nefazodone [333].

Agonist medications share pharmacological mechanisms of action with the abused drug, thereby producing some common neurochemical effects. Agonist medications for treatment of cocaine dependence have included direct dopamine receptor agonists and indirect dopamine receptor agonists. Preclinical studies in nonhuman primates involving chronic treatment with direct dopamine receptor agonists on cocaine self-administration have not yielded encouraging results. For example, chronic treatments with full and partial dopamine D₁ receptor agonists produced nonselective decreases in cocaine- and food-maintained responding in squirrel monkeys [334] or moderately selective decreases in cocaine-maintained responding in rhesus monkeys [335]. The D₂/D₃ receptor agonist quinpirole failed to reliably suppress cocaine self-administration at doses that produced overt toxicity in squirrel monkeys

[336]. Clinical studies with dopamine receptor agonists have also been disappointing. For example, bromocriptine is a D₂-like receptor agonists and a partial D₁-like receptor agonist used mainly in the treatment of Parkinson's disease. In a human laboratory study, pretreatment with bromocriptine prior to cocaine administration had no effect on cocaine-induced euphoria [337]. Moreover, the results of outpatient clinical trials with bromocriptine were inconclusive [316]. A recent eight-week open-label study with combined bupropion and bromocriptine in cocaine-dependent subjects did not find improvement based on cocaine-positive urine screens [338]. Collectively, these findings do not support the use of bromocriptine as a pharmacotherapy for cocaine dependence.

Studies evaluating the effects of indirect dopamine agonists have yielded mixed but more encouraging results. Mazindol, a dopamine and norepinephrine reuptake inhibitor used in the treatment of obesity, did not alter the subjective effects of cocaine in a human laboratory study [339]. Moreover, in a six-week, placebo-controlled study in cocaine-dependent subjects, mazindol did not differ from placebo in reducing cocaine use and mazindol treatment was not well tolerated [340]. Methylphenidate, a dopamine and norepinephrine reuptake inhibitor used in the treatment of ADHD and narcolepsy, was well tolerated and led to better retention than placebo but was not effective in reducing cocaine use in cocaine-dependent subjects [341]. In a separate study in cocaine-dependent subjects with ADHD, there was no significant reduction in cocaine use [342]. However, clinical studies with the indirect dopamine agonist disulfiram have been more encouraging. Disulfiram blocks the conversion of dopamine to norepinephrine by inhibiting the enzyme dopamine β -hydroxylase, thereby increasing brain dopamine concentrations. Two controlled clinical trials in cocaine addicts that were not alcoholics found disulfiram to be significantly better than placebo in promoting cocaine abstinence [322, 323]. A recent outpatient study in cocaine-dependent subjects replicated these earlier findings, showing that disulfiram reduced cocaine use more than placebo did [324]. Collectively the results suggest that disulfiram may be effective in treating cocaine addicts, including those who are not alcoholic.

There is growing support from preclinical studies in nonhuman primates and recent clinical studies for the use of stimulant medications in the treatment of cocaine dependence [341, 343–346]. A number of studies in nonhuman primates provide evidence that dopamine transporter inhibitors can effectively attenuate cocaine self-administration [21, 23, 37, 38, 347]. Hence, the development of compounds that target the dopamine transporter represents a logical approach for the pharmacological treatment of cocaine dependence. Similarly, chronic treatment with the nonselective monoamine releaser dextroamphetamine produced sustained and selective decreases in cocaine self-administration in rhesus monkeys [344, 345]. A possible limitation to the use of dopamine transport inhibitors and monoamine releasers as medications for the treatment of cocaine dependence is their potential for abuse, given their documented reinforcing effects. However, recent evidence suggests that the reinforcing effectiveness of dopamine transporter inhibitors may be limited by dual actions at the dopamine and serotonin transporters. For example, a cocaine analog with high affinity at dopamine and serotonin transporters was not reliably self-administered when substituted for cocaine, yet suppressed cocaine self-administered at low levels of dopamine transporter occupancy [38]. Similarly, monoamine-releasing agents exhibited decreasing reinforcing efficacy when the serotonin-releasing potency was

increased relative to the dopamine-releasing potency [46]. Accordingly, combined actions at dopamine and serotonin transporters may enhance effectiveness in reducing cocaine use and limit the abuse liability of the medication. Importantly, compelling data have emerged from clinical research supporting indirect agonist pharmacotherapy for stimulant abuse and dependence. Well-designed, placebo-controlled clinical trials in cocaine-dependent subjects found that sustained-released dextroamphetamine was better than placebo at reducing cocaine intake [314].

Medications that target glutamatergic function are reasonable candidates given the involvement of glutamatergic circuits in reward-related brain regions and evidence of cocaine-induced dysregulation of glutamate function [348]. Modafinil, recently approved for the treatment of narcolepsy, enhances glutamate function via unidentified mechanisms that induce increases in glutamate synthesis and striatal glutamate brain levels [349]. Interestingly, modafinil has clinical effects in nondependent subjects that are opposite to the cocaine withdrawal syndrome [350]. In patients with severe cocaine withdrawal symptoms, modafinil treatment resulted in higher rates of cocaine abstinence and treatment retention. In a separate study, the subjective effects of cocaine administration in cocaine-dependent subjects were significantly reduced [351]. Modafinil was well tolerated in both studies and is currently being investigated for treatment of cocaine dependence in large, controlled clinical studies.

Recently, the GABAergic system has received significant attention as a potential target for the pharmacological treatment of cocaine dependence [315]. For example, baclofen is an antispasticity agent that is a nonselective GABA_B agonist. In a placebo-controlled study in cocaine-dependent subjects, baclofen treatment enhanced cocaine abstinence compared to placebo [352]. Tiagabine is an antiepileptic medication that increases synaptic levels of GABA by inhibiting GABA transporters. A placebo-controlled pilot study in opioid-dependent patients maintained on methadone reported that tiagabine attenuated cocaine use [353]. Topiramate is another antiepileptic medication that potentiates GABAergic transmission, but it has a complex pharmacology that includes antagonism of AMPA/kainate glutamate receptors [354]. In a recent placebo-controlled pilot study in cocaine-dependent subjects, topiramate treatment enhanced cocaine abstinence [355]. Collectively, these initial studies suggest that the GABAergic systems may be a useful pharmacological target for cocaine medication development, although additional, larger scale clinical trials are clearly warranted.

16.5 SUMMARY

The abuse liability of psychostimulants is well established and represents a significant public health concern. Currently, no effective pharmacotherapy for psychostimulant abuse has demonstrated efficacy for long-term use. A better understanding of the neuropharmacological effects of cocaine and related psychostimulants has supported efforts to develop and improve useful pharmacotherapies for psychostimulant use and dependence. An extensive literature documents the critical importance of the monoamines in the behavioral pharmacology and addictive properties of psychostimulants. In particular, dopamine plays a primary role in their reinforcing effects and abuse liability. The relevance of the dopamine transporter in the reinforcing

effects of cocaine is supported by numerous preclinical studies of drug self-administration and, more recently, by nonhuman primate and human neuroimaging studies. Also, a growing literature indicates that the serotonergic and noradrenergic systems can effectively modulate the neurochemical and behavioral effects of cocaine and amphetamine. Similarly, cortical glutamatergic systems provide important regulation of dopamine function, and GABAergic systems provide inhibitory neuromodulation of monoaminergic and glutamatergic function. Psychostimulants also activate the HPA axis and thereby engage neuroendocrine systems linked to stress reactivity. Lastly, endogenous neuropeptide systems appear to play an important role in the neuropharmacology and addictive properties of psychostimulants. Repeated exposure to psychostimulants can lead to robust and enduring changes in all of these neurobiological substrates, resulting in altered sensitivity to acute drug effects on neurochemistry and behavior as well as dysregulation of brain function linked to dependence and addiction. Recent approaches in medication development to treat psychostimulant abuse and dependence have focused largely on these well-established neurobiological mechanisms with some degree of success. In particular, functional agonist treatments may be used effectively to stabilize neurochemistry and behavior. Similarly, medications that target glutamatergic and GABAergic function are reasonable candidates that have received significant attention, and some have demonstrated effectiveness in attenuating cocaine use and enhancing cocaine abstinence. However, these encouraging results will require additional clinical studies in order to identify safe and efficacious pharmacotherapies.

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MDMA AND OTHER “CLUB DRUGS”

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17.1	MDMA	614
17.1.1	Introduction	614
17.1.2	Reinforcing Properties	614
17.1.3	Acute Biochemical and Behavioral Effects	616
17.1.3.1	Monoamine Release in Brain	616
17.1.3.2	Tryptophan Hydroxylase	617
17.1.3.3	Effect on Neurotransmitter Receptors and Transporters	617
17.1.3.4	Effect on Body Temperature	617
17.1.3.5	Neuroendocrine and Immune Responses following MDMA	618
17.1.3.6	Effects on Behavior	618
17.1.4	Long-Lasting Biochemical and Behavioral Effects: Neurotoxicity	619
17.1.4.1	Long-Term Neurochemical Changes	619
17.1.4.2	Experimental Studies with Similar Doses to that Consumed by Humans	620
17.1.4.3	Mechanisms of Neurotoxicity	621
17.1.4.4	Functional Consequences of Neurotoxic Lesion	629
17.1.5	Biochemical and Functional Changes in Human Brain	631
17.2	GHB	632
17.2.1	Pharmacological Effects	633
17.2.2	Mechanism of Action	634
17.2.3	GHB and Addiction	635
17.3	Ketamine	636
17.3.1	Pharmacological Effects	636
17.4	Flunitrazepam (Rohypnol)	637
17.4.1	Pharmacological Effects	637
17.5	LSD	637
17.5.1	Pharmacological Effects	638
17.5.2	Mechanism of Action	638
17.5.3	Hallucinogens and Addiction	639
17.6	Summary	640
	References	640

17.1 MDMA

17.1.1 Introduction

Gary Henderson, a pharmacist from the University of California, first coined the term “designer drugs” in the 1960s. Its purpose was to encompass substances of synthetic origin which were structurally and pharmacologically similar to existing substances (widely used illegal drugs) but which, due to their chemical novelty, had not been specifically listed and therefore escaped legal control. One group to appear on the scene is the methylenedioxy derivatives of amphetamine and methamphetamine, and of these, perhaps the best known and probably one of the most widely abused member is 3,4-methylenedioxymethamphetamine (MDMA), popularly known as “Ecstasy.”

The first recorded mention of the compound is in a German patent filed by E. Merck in 1912 and granted in 1914, where it appears as a possible precursor of pharmacologically active compounds. The 1980s saw the rapid establishment of MDMA as a recreational drug of abuse. It became popular with North American university students among whom it came to be known under a number of different names: XTC, Adam, MDMA, M&M.

Concurrently, low-dose MDMA [75–175 mg, by mouth (p.o.)] was being used as an adjuvant in psychotherapy due to the apparent ability of the drug to reduce anxiety and psychological defense barriers, increase self-confidence, and facilitate communication between therapist and patient.

In 1985, the U.S. Drug Enforcement Administration (DEA) placed MDMA in Schedule I of the Controlled Substances Act due to its high potential for abuse, lack of clinical application, disagreement among experts as to its safety under medical supervision, and evidence that 3,4-methylenedioxyamphetamine (MDA), a related compound and principal metabolite of the drug, produced degeneration of serotonergic terminals in rat brain.

The drug is taken orally in the form of tablets and generally consumed at weekends, often at dance clubs, or “raves.” Ecstasy tablets are available in a wide variety of colors, shapes, and sizes, each decorated with a different design or logo. It produces a series of subjective symptoms and sensations among which the most frequent include an increase in empathy, that is, a sense of closeness to other people, an emotional opening up, an increased ability and willingness to communicate, a reduction in negative thoughts and inhibitions, a heightened sense of perception of sound, color, and touch, an increase in locomotor activity, insomnia, and an increase in alertness. These effects appear in the first 20–60 min after a single dose, reaching a maximal effect after 60–90 min and lasting 3–5 h.

17.1.2 Reinforcing Properties

There have been several studies on the rewarding properties of MDMA, and these studies generally implicate dopamine in the actions of the drug. Systemic MDMA administration has been shown to increase extracellular dopamine in the mesolimbic forebrain, namely the nucleus accumbens (NAc) [1–3]. The NAc is responsible for the incentive motivational properties of most drugs of abuse, and the rewarding effects of MDMA have been shown using the appropriate paradigms. Thus, rats treated with MDMA developed a positive and dose-dependent response in the conditioned place

preference (CPP) test [2, 4–6]. Since CPP is believed to be a measure of appetitive behavior, where the animal associates contextual cues with either a positive or negative feeling produced by the drug, these results provide direct evidence of the rewarding properties of MDMA in rats. A rewarding effect of MDMA has also been shown in the self-stimulation paradigm in rats [7], where MDMA lowers the reward threshold of electrical stimulation, and in the drug self-administration test in rats [8, 9] and baboons [10]. The locomotor hyperactivity observed after MDMA injection is also consistent with this drug exerting a positive rewarding effect [11, 12]. In order to determine whether dopaminergic mechanisms mediate the reinforcing effects of MDMA, Daniela and coinvestigators [9] have evaluated the effects of the D₁ receptor antagonist 7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH 23390) on MDMA self-administration and compared them to the effects on MDMA-induced hyperactivity. SCH 23390 pretreatment produced a dose-dependent decrease in MDMA-induced hyperactivity and a rightward shift in the dose-response curve for MDMA self-administration. These data indicate that dopamine plays a key role in the hyperactivity that follows MDMA injection and suggest that MDMA self-administration, like the self-administration of other drugs of abuse, is dependent on the activation of dopaminergic substrates.

Recently, it has been suggested that the rewarding effects of MDMA are more pronounced at high ambient temperature and that the enthusiasm of recreational users for consuming the drug in hot environments might not be coincidental. It has been shown that an elevation of ambient room temperature enhanced the prosocial effects of MDMA and the number of MDMA infusions self-administered by rats [13]. In addition, the neurochemical changes related to rewarding effects of MDMA are more pronounced when the drug is given at high ambient room temperature [14]. By means of *in vivo* microdialysis, it has been shown that elevation of ambient temperature enhances the effect induced by low and medium doses of MDMA on dopamine release in the shell of the NAc, but not the striatum, of freely moving rats. The output of serotonin (5-HT) is also enhanced in the NAc, but not the striatum, by high ambient temperature conditions. Taken together, it seems reasonable to propose that the enhanced mesolimbic dopamine release seen at high ambient temperature is responsible for the changes seen by Cornish and coinvestigators [13].

It is also possible that previous exposure to MDMA may increase the reinforcing effects of other psychostimulants such as cocaine and amphetamine. There is strong experimental evidence indicating that rats treated with MDMA subsequently develop enhanced behavioral and biochemical responses to psychomotor stimulants. Studies have shown that in animals pretreated with MDMA cocaine produces a greater increase in the extracellular levels of dopamine in the NAc than that seen in control rats [15]. If the reinforcing effect of cocaine is dependent, in part, upon increased dopamine release in the NAc, these data suggest that MDMA may increase vulnerability to cocaine abuse. Subsequently, using the appropriate paradigms, it has been shown that rats pretreated with MDMA show a significantly greater CPP response to cocaine than vehicle-treated animals [16] although this may depend on the protocol of MDMA administration since another study failed to confirm this [17]. In mice, adolescent exposure to MDMA increased hyperlocomotion and facilitated the reinstatement of cocaine-seeking behavior, as assessed by CPP after a 14-day drug-free period [18]. These results provide direct evidence of an increase in

the rewarding properties of cocaine in rats preexposed to MDMA and suggest that MDMA abuse leads to increased vulnerability to cocaine addiction and dependence. In fact, preexposure to a high dose of MDMA facilitates acquisition of cocaine self-administration in rats [19], which again confirms that MDMA preexposure sensitizes rats to the reinforcing effects of cocaine.

The effect of MDMA on the acquisition of intravenous amphetamine self-administration and the reinstatement of amphetamine-seeking behavior by either MDMA or amphetamine has been recently evaluated [20]. Preexposure to a 5-HT-depleting regimen of MDMA slows the acquisition of amphetamine self-administration but may sensitize animals to the locomotor stimulating and priming effects of MDMA on drug-seeking behavior. Therefore, it seems likely that MDMA exposure may result in an enhanced sensitivity to psychostimulants and that this is a vulnerability to relapse that may be rather long-lasting.

Consequently, and although we must be extremely cautious when extrapolating results from animals to humans, it would be reasonable to propose that MDMA users may be at greater risk of developing addiction and dependence to other psychomotor stimulants.

17.1.3 Acute Biochemical and Behavioral Effects

17.1.3.1 Monoamine Release in Brain. MDMA is an amphetamine compound, and therefore, like other compounds of this class, its primary property is the release of monoamines in the brain.

MDMA administration produces an acute rapid release of 5-HT in the rat brain. This has been demonstrated both by in vivo microdialysis [21–23] and the decreased concentration of 5-HT in cerebral tissue taken from treated animals [24–26]. These references reflect only a few of the many studies that have reported these changes. The release occurs in all the major forebrain regions [27] and is inhibited by prior administration of 5-HT selective uptake blockers such as fluoxetine [23]. MDMA-induced 5-HT release can also be demonstrated in vitro by using brain slices [28] or synaptosomal preparations [29].

MDMA is also a potent compound in inducing dopamine release, and again this has been demonstrated using in vivo microdialysis [1, 30, 31] and in vitro using cerebral tissue slices [28].

The role of the dopamine uptake site in MDMA-induced dopamine release is controversial [32], and Mehan and coinvestigators [23] have suggested that MDMA may enter the dopamine terminal primarily by diffusion rather than via the uptake carrier site.

The role of 5-HT release in inducing dopamine efflux is indicated by the fact that pretreatment with fluoxetine attenuates MDMA-induced dopamine release in the striatum [33] and administration of a 5-HT agonist will enhance dopamine release [30]. Since ritanserin attenuates MDMA-induced dopamine release [22], it is reasonable to propose that MDMA can modify dopamine release by enhancing the efflux of 5-HT, which then acts on 5-HT_{2A/2C} receptors.

While in vitro evidence suggests that MDMA can enhance norepinephrine release from brain tissue [34] and from synaptosomes [35], in vivo microdialysis data are presently lacking.

17.1.3.2 Tryptophan Hydroxylase. MDMA administration inhibits the activity of tryptophan hydroxylase in the brain [36–38] and this effect is very rapid [37]. Inhibition is still observable two weeks after a single dose of MDMA [39]. Since tryptophan hydroxylase is the rate-limiting enzyme involved in 5-HT synthesis, this indicates that a proportion of the 5-HT loss seen soon after MDMA administration may be due to hydroxylase inhibition rather than release, while the long-term decrease in cerebral 5-HT content following MDMA may be due in part to inhibition of the enzyme rather than neurotoxicity (see later).

Since MDMA has no direct inhibitory action on the enzyme *in vitro* [39], it is possible that the inhibition seen *in vivo* results from the action of an MDMA metabolite, an hypothesis supported by the finding that the MDMA quinone derivative can inactivate the enzyme [40].

17.1.3.3 Effect on Neurotransmitter Receptors and Transporters. MDMA binds to the 5-HT, dopamine, and norepinephrine transporters, although its affinity for the 5-HT site is at least 10 times higher than for the other monoamine transporter sites [41, 42].

MDMA binds with a 1–10 μM affinity for 5-HT₂, α -adrenergic, M₁ muscarinic, and H₁ histaminic sites. Other sites (including 5-HT₁) are in the 10–100 μM range while dopamine D₁ and D₂ sites are > 100 μM [42].

17.1.3.4 Effect on Body Temperature. The effect of MDMA on body temperature has been investigated extensively in experimental animals, partly because there is a clear clinical correlate.

MDMA produces a rapid hyperthermic response in rats housed in normal or warm room conditions ($\leq 20^\circ\text{C}$) with a peak of 1–2 $^\circ\text{C}$ above normal rectal temperature [43–46]. The response is dose dependent [47]. When the rats are present in a cool ambient room temperature ($< 19^\circ\text{C}$), the effect of MDMA is to produce a hypothermic response [45, 48, 49].

If the rats are in a warm environment, the MDMA-induced hyperthermia is greater than in a normal environment [48, 50] (Fig. 17.1). Furthermore, giving divided doses over several hours to mimic “binge dosing” (but with the same total dose administered) results in a greater peak response than after a single dose [50] (Fig. 17.1).

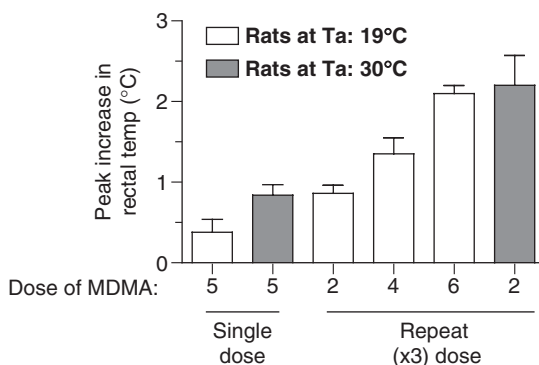


Figure 17.1 Effect of single or repeated doses (binge dosing) of MDMA (mg/kg) on peak rectal temperature increase in Dark Agouti rats housed at ambient temperature (T_a) of either 19 or 30 $^\circ\text{C}$. Doses of MDMA are shown.

The rise in rectal temperature appears to result from the action of the dopamine released by MDMA, on dopamine D₁ receptors, since the effect was antagonized by the D₁ antagonist SCH 23390, but not the D₂ antagonist remoxipride [23]. However, the hypothermia seen after MDMA is given to rats in a cool environment is dopamine D₂ receptor mediated, since remoxipride blocks the effect while SCH 23390 is without effect (Green and coinvestigators, unpublished).

Gordon and coinvestigators [51] found that the metabolic rate increased when MDMA was given to rats housed at 20°C, together with an increase in evaporative water loss. This suggests increased thermogenesis. This interpretation is supported by the fact that there is an involvement of the hypothalamic–pituitary–thyroid axis in the response [52] and crucially that β_3 adrenoceptors are involved, which indicates an action in brown adipose tissue [53]. Heat loss mechanisms do not appear to be activated immediately to compensate for the thermogenesis, since the tail temperature does not rise following MDMA [23] (the tail is a major heat loss organ in the rat [54]).

It has been known for many years that grouping mice increases the hyperthermic effect of amphetamine and increases its toxicity compared to the effect of the drug when given to individually housed animals [55]. Recently, Fantegrossi and coinvestigators [56] observed that the same phenomenon occurred when MDMA was examined in both singly housed and grouped mice. This has implications for the recreational use of MDMA in humans since the drug is primarily used in hot, crowded club conditions.

17.1.3.5 Neuroendocrine and Immune Responses following MDMA. The monoamine release by MDMA initiates neuroendocrine changes. In rats both serum corticosterone and prolactin concentrations increase after MDMA [43], with the corticosterone response lasting over 4 h and the prolactin more than 1 h. The corticosterone response was dose dependent and blocked by ketanserin, suggesting an involvement of 5-HT.

Both aldosterone and renin also increase after MDMA administration to rats, and again, the aldosterone rise appears to be induced through increased 5-HT release [57]. In vitro studies have also demonstrated MDMA-induced oxytocin and vasopressin release in hypothalamic tissue [58, 59].

Several studies have demonstrated that MDMA can alter the immune function of the rat. Leucocyte counts are reduced for several hours after MDMA, as is concanavalin A-induced lymphocyte proliferation. This suggests that the drug produces a sustained suppression of mitogen-stimulated lymphocyte proliferation as well as leucocyte count and indicates a suppression of immune function [60].

17.1.3.6 Effects on Behavior. Since MDMA releases 5-HT from the nerve ending, it is not surprising that it can produce aspects of the *serotonin behavioral syndrome* [44, 61–63]. This syndrome was first described by Grahame-Smith [64] and consists of head weaving, reciprocal forepaw treading, proptosis, piloerection, and salivation. MDMA also produces a dose-dependent increase in locomotor activity [65], which can be antagonized by N-[4-methoxy-3-(4-methyl-1-piperazinyl)]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4 carboxamide hydrochloride monohydrate (GR127935), a 5-HT_{1B/1D} antagonist, but not by N-(2-[4-(2-methoxy-phenyl)-1-piperazinyl] ethyl)-N-(2-pyridinyl) cyclohexane trihydrochloride (WAY100635), a 5-HT_{1A}

antagonist. However, the work of Bankson and Cunningham [66] has emphasized the complexity of the hyperactivity response by showing the possible involvement of 5-HT_{2B/2C} receptor function. Acute MDMA administration has been examined in various tests of anxiety-like behavior and data still remain somewhat conflicting. In the elevated-plus maze test Morley and McGregor [67] found the drug to produce an anxiogenic-like effect in rats. However, in the social interaction test an anxiolytic-like effect was seen. In contrast, Bhattacharya and coinvestigators [68] observed an anxiogenic effect in both tests. Lin and coinvestigators [69] saw an anxiogenic effect in mice exposed to the elevated-plus maze following low doses of the drug but an anxiolytic effect following high-dose administration. An anxiogenic effect was also reported to occur following MDMA administration to mice when the social interaction test was used [70].

17.1.4 Long-Lasting Biochemical and Behavioral Effects: Neurotoxicity

MDMA produces a characteristic biphasic neurochemical response which can be divided into acute effects, consisting of changes in cerebral biochemistry which are reversible, and long-term effects, including biochemical and structural changes in the brain which are persistent and are considered to reflect neurotoxicity.

MDMA administration produces a marked and rapid (3–4-h) depletion of 5-HT in several brain regions [71]. There is a recovery of brain 5-HT concentration within 24 h, but this is followed within three to four days by a long-term decrease which is unequivocal and which lasts for several months [71, 72]. This second phase of monoamine loss reflects neurodegenerative changes in serotonergic nerve terminals that have occurred, and it has been demonstrated in rats, guinea pigs, and several species of nonhuman primates. In mice the response is quite different, for while MDMA also behaves as a neurotoxin, it produces a reduction in the number of dopaminergic nerve terminals in striatum but leaves 5-HT-containing neurons intact. It is worth emphasizing that neurotoxicity in mice requires repeated injection of MDMA and also higher doses than those used in rats.

17.1.4.1 Long-Term Neurochemical Changes. There is clear and unequivocal evidence for a substantial and sustained long-term neurotoxic loss of 5-HT nerve terminals in several regions of the rat brain [31, 71, 73–77]. The degeneration has been demonstrated histologically [78, 79] and biochemically and is reflected in a substantial decrease in tryptophan hydroxylase activity; a reduction in the concentration of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA); a decrease in the density of 5-HT uptake sites labeled with [³H]paroxetine [77, 80–82]; and a reduction in the immunoreactivity of fine 5-HT axons in the neocortex, striatum, and hippocampus [78]. The dose required to induce neurotoxicity in rats is strain dependent. For example, Dark Agouti rats require a single dose (10–15 mg/kg) of MDMA to produce a clear 30–50% or greater loss in cerebral 5-HT content [47, 83] while strains such as Sprague–Dawley, Hooded-Lister, and Wistar require repeated and higher doses of MDMA (often 20 mg/kg or more) [44, 63, 84].

Although the neurotoxic potential of MDMA is well established, there is relatively little information on the fate of lesioned 5-HT neurons. Some authors have suggested that there is a substantial recovery of 5-HT function after MDMA-induced lesions in rodents [85–90]. While rats exposed to repeated administration of MDMA (10 mg/kg)

show a marked reduction (60–80%) of 5-HT transporters in the hippocampus, striatum, and cerebral cortex 2 weeks after dosing, some signs of recovery are observed in all brain areas at 16 weeks, and by 32 weeks the recovery is complete in the striatum and cerebral cortex [88]. Immunohistochemical data confirm these biochemical results and show that the recovery of 5-HT presynaptic markers is due to the regeneration of 5-HT-containing axons [79, 88]. Axonal reinnervation refers to fine 5-HT axons coming from the dorsal raphe nucleus, which are the axons selectively lesioned by MDMA [78, 91], while thicker and varicose axons coming from medial raphe nucleus are not affected by MDMA [78, 91] and do not show any evidence of collateral ramification [79]. Nevertheless, the pattern of reinnervation is anomalous, since distal brain areas such as the neocortex remain denervated while proximal areas such as the hypothalamus appear either reinnervated or hyperinnervated [92]. Together, these results confirm the plasticity of central 5-HT neurons [93, 94] and indicate that under specific conditions 5-HT neurons have recovered from the MDMA-induced damage. Nevertheless, although the effects of MDMA in rodents seem to be partially reversible, they are probably permanent in primates. Insel and coinvestigators [95] observed significant 5-HT deficits in the hippocampus, striatum, and cortex of the *Maccacus rhesus* 14 weeks after repeated administration of MDMA. Similar results have been found in the hippocampus, caudate nucleus, and frontal cortex of squirrel monkeys 18 months after dosing [96]. In a study carried out seven years after MDMA dosing, Hatzidimitriou and coinvestigators [97] found that 5-HT axon density is only 50–65% of control values in neocortical regions. These data suggest that the 5-HT axons in nonhuman primates do not recover from MDMA-induced damage, and this information is particularly significant since it could be indicative of what happens in human beings, considering that the dose given to nonhuman primates is similar to that taken by humans.

17.1.4.2 Experimental Studies with Similar Doses to that Consumed by Humans. Recreational Ecstasy users tend to believe that the doses administered to experimental animals to induce toxicity are much higher than those that they use and that the data obtained are not relevant to human use. They therefore conclude that MDMA is a safe drug. Ecstasy tablets have been reported to contain generally between 70 and 150 mg [98], which is equivalent to 1–2 mg/kg of MDMA in a 70-kg human. However, Ecstasy is often ingested repeatedly in such a way that it is frequent for an individual to consume several tablets during a weekend [99]. What is perhaps most important is the *exposure* to the drug, and measurement of plasma levels is particularly important for that assessment. However, this measurement is rarely made in experimental animals. Nevertheless, the study of Colado and coinvestigators [83] did show that a single dose of MDMA of 10 mg/kg administered to Dark Agouti rats produced a plasma level of MDMA of near 10 nmol/mL 45 min later. Similar values have been observed clinically in overdose cases. This therefore suggests that dosing schedules in rats are often producing exposure that is not markedly different from those experienced by recreational users.

Several studies have been performed modeling the doses and dosing schedules of MDMA often used by human recreational users. O'Shea and coinvestigators [47] administered MDMA to Dark Agouti rats at the dose of 4 mg/kg once or twice daily for four consecutive days. The rats were killed seven days after dosing. Once-daily injection had no effect on regional brain concentration of 5-HT and 5-HIAA, while

twice-daily administration resulted in a substantial depletion in all brain areas examined. These data thus indicate that high or frequent doses of MDMA are required to produce neurotoxic damage.

Studies giving low and repeated doses of MDMA have also been performed in nonhuman primates, and these showed that neurotoxic effects are more pronounced than those observed in rodents [100]. For example, administration of 2.5 mg/kg MDMA twice daily for four consecutive days produced 44% depletion of 5-HT in the somatosensory cortex of the monkey [101], while to obtain similar depletion in the brain of Dark Agouti rats, it is necessary to administer 4 mg/kg MDMA when following the same dosing protocol [47].

The degree of 5-HT depletion may also be dependent upon the route of administration, although data on this point are conflicting. While similar neurotoxic effects were observed in rats following oral or subcutaneous dosing [102], oral administration in monkeys seemed to be less effective than subcutaneous injection [103]. In contrast, Kleven and coinvestigators [104] obtained a more marked effect of the drug when it was given to rhesus monkeys by the intragastric route rather than subcutaneously.

A practice sometimes employed by recreational users of Ecstasy is “binge” dosing, comprising the ingestion of several doses on a single occasion [105, 106]. This type of dosing has been recently modeled by administering three doses of MDMA (2, 4, or 6 mg/kg) 3 h apart and evaluating its consequences on the long-term neurotoxic effects of MDMA when administered to rats housed either at 19°C or in a room kept at 30°C to simulate the way in which the drug is sometimes taken by human beings in a hot, dance club environment [107]. At 19°C MDMA produced a dose-dependent long-term loss of 5-HT in several brain regions with an approximate 50% loss following 3×4 mg/kg and a 65% decrease following 3×6 mg/kg. When MDMA at the dose of 4 mg/kg was injected repeatedly to rats housed at 30°C, a larger long-term 5-HT depletion (65%) than that found in rats treated at 19°C was observed, the effect being similar to that produced by the dose of 6 mg/kg given at 19°C ambient temperature.

17.1.4.3 Mechanisms of Neurotoxicity.

17.1.4.3.1 Neurotoxic Metabolite. The toxicity induced by MDMA in both rat and mouse is mediated by one or more metabolites of the drug that have been formed peripherally. Evidence for this in rats stems from the fact that central administration of MDMA does not produce reductions in the concentrations of 5-HT in various terminal fields [108–110] in spite of brain concentrations being greater than those following systemic administration of a neurotoxic dose of the drug [110]. Central administration of the MDMA did, however, produce an increase in 5-HT release in the hippocampus, and dopamine release in the striatum, similar to that produced by systemic administration [31, 110], suggesting that whereas the neurotoxic effects of the drug are produced by a peripherally formed metabolite, the acute effects are induced by the parent compound.

In contrast to that which occurs in the rat, recent studies indicate that in mice MDMA is responsible for neither the acute nor the long-term effects of the drug [111]. Intrastratial administration of MDMA does not reproduce the reduction in dopamine and the changes in 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) observed following systemic administration.

The lack of neurotoxicity following the central administration of the drug has led to numerous studies on the peripheral metabolism of MDMA, mostly in the rat. One

of the principal metabolites of MDMA in this species is MDA, the product of N demethylation via the CYP1A2 isoform of the cytochrome P450 monooxygenase system. This compound exhibits a similar profile but higher potency as a serotonergic neurotoxicant than MDMA and, like MDMA, is not toxic when injected directly into the brain [108]. Thus, MDA may be an intermediate product but is not the final neurotoxic metabolite.

The drug can undergo various other routes of metabolism (O demethylation, deamination, and hydroxylation) and conjugation (O methylation, O glucuronidation, and O sulfation), giving rise to 17 in vivo metabolites [112–114], several of which have been tested in vivo. None, however, has been found to produce the same pattern of toxicity as that produced by systemic MDMA. Thus, aromatic hydroxylation gives rise to 6-hydroxy-MDMA [114, 115], which is demethylated by CYP2D1 to yield 2,4,5-trihydroxymethamphetamine [112, 113], both of which have been shown to produce a different profile of toxicity to MDMA—either lack of toxic effect or toxicity of both the 5-HT and dopamine neurotransmitter systems, respectively [116, 117].

Direct demethylation of MDMA or MDA by CYP2D1 gives rise to 3,4-dihydroxymethamphetamine (HHMA; *N*-methyl- α -methyldopamine, *N*-Me- α -Me DA) and its demethylated equivalent, 3,4-dihydroxyamphetamine (HHA; α -methyldopamine, α -Me DA), which are unstable reactive catechol derivatives that may in turn form quinones that can auto-oxidize and produce free radicals. Alternatively, these catechols can undergo O methylation at position 3 or 4 of the aromatic ring to form 3-hydroxy-4-methoxy(meth)amphetamine [4-*O*-methyl- α -(methyl)dopamine, 4-*O*-Me- α -(Me) DA] or 4-hydroxy-3-methoxy(meth)amphetamine [3-*O*-methyl- α -(methyl)dopamine, 3-*O*-Me- α -(Me) DA]. Neither α -Me DA nor 3-*O*-Me- α -Me DA produce serotonergic toxicity [118].

In recent years, the glutathione-formed conjugates of the quinones that are discussed above have been suggested as possible neurotoxic metabolites of the drug [119, 120]. Thus, a putative metabolite adduct, 5-(glutathion-*S*-yl)- α -Me DA, crosses the blood–brain barrier by means of the glutathione carrier [121] and is neurotoxic to 5-HT terminals following repeated intracerebral injection [119]. Addition of a further glutathione molecule to 5-(glutathion-*S*-yl)- α -Me DA yields 2,5-(glutathion-*S*-yl)- α -Me DA, which is a more potent neurotoxin [119]. Once in the brain these compounds can be further metabolized to cysteine or acetylcysteine adducts which are more potent toxins [119, 120, 122]. Jones and coinvestigators [122] recently identified glutathione and *N*-acetyl cysteine conjugates of *N*-Me- α -Me DA in rat striatal dialysate after subcutaneous MDMA administration. Furthermore, inhibition of γ -glutamyl transpeptidase increases the toxicity produced by MDMA and MDA and the levels of the *N*-acetyl cysteine adducts in brain by increasing the pool of the thioether compounds available for transport by the glutathione carrier [121, 122].

In the mouse, no studies have been carried out on products of metabolism, although Escobedo and coinvestigators [111] found that the major compound in brain following peripheral MDMA administration is MDMA itself. This is in sharp contrast to what is observed in rats, where MDA is the major metabolite at low doses, and in humans, where HHMA predominates [123]. Further studies on the different profiles of metabolism in the rat and mouse are required in order to evaluate if these differences could account for the neurotoxic selectivity of the drug in the different animal species.

17.1.4.3.2 Oxidative Stress. There is much experimental evidence indicating that a major mechanism by which MDMA induces damage to 5-HT-containing neurons in the rat brain is by inducing an oxidative stress process [77, 124] which is ultimately reflected by an increase in lipid peroxidation in several brain areas [125]. Two factors contribute to this process, a decrease in the antioxidant capacity of the brain reflected as a reduction in vitamin E and ascorbate levels [126] and an increase in the formation of hydroxyl-free radicals [77, 127]. The use of intracerebral microdialysis has demonstrated that systemic administration of MDMA increases the formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) from salicylate in hippocampal and striatal dialysates of rats and mice [77, 128–130], a conversion that only occurs in the presence of a high concentration of hydroxyl-free radicals. Administration of the hydroxyl radical scavenger α -phenyl-*N*-*tert*-butyl nitron (PBN) abolished the MDMA-induced rise in 2,3-DHBA and attenuated neurotoxic damage and did so without altering MDMA-induced hyperthermia [77, 127]. Other free-radical scavenging drugs have also been found to protect against MDMA-induced damage. Thus, administration of large doses of sodium ascorbate or L-cysteine 30 min prior to and 5 h following MDMA injection prevented the long-term depletion of 5-HT induced by MDMA [131]. Ascorbic acid administration also suppressed hydroxyl radical formation in the striatum [126]. The metabolic antioxidant α -lipoic acid injected repeatedly (up to two days after MDMA) also totally prevented the reduction in the number of 5-HT transporters observed seven days after MDMA dosing [132]. In addition to hydroxyl radicals, nitrogen-reactive species are also involved in MDMA-induced neurotoxicity. Gudelsky's team [133] found evidence for the involvement of nitric oxide in MDMA-induced serotonergic neurotoxicity. A multiple-dose regimen of MDMA produced a significant increase in levels of nitrotyrosine and nitric oxide in the striatum. Pretreatment with the neuronal nitric oxide synthase (NOS) inhibitor *S*-methyl-L-thiocitrulline (*S*-MTC) afforded neuroprotection against MDMA-induced 5-HT depletion without attenuating MDMA-induced hyperthermia. In addition, administration of NOS inhibitors, as well as a peroxynitrite decomposition catalyst, attenuated the long-term depletion of striatal 5-HT and dopamine depletion produced by the local perfusion of MDMA and malonate.

In mice there is also evidence for a key role of oxygen- and nitrogen-reactive species in the MDMA-induced neurotoxicity on dopamine neurons. Pretreatment with either of the NOS inhibitors *S*-MTC and *N*-(4-(2-((3-chlorophenyl methyl)amino)-ethyl)-phenyl) 2-thiopene carboxamidine hydrochloride (AR-R17477AR) provided significant neuroprotection against the long-lasting MDMA-induced dopamine depletion, and AR-R17477AR prevented the rise in 2,3-DHBA levels in striatal dialysates of the mice [129]. Also supporting the suggestion that free-radical formation is responsible for MDMA-induced neurotoxicity is the study in which mice fed a selenium-deficient diet showed an enhancement of the long-term dopamine depletion and, strikingly, also a long-term loss of 5-HT content in several brain areas, an effect not observed in mice maintained with a standard diet [134]. This effect is due to an impairment of the antioxidant detoxification system mediated by glutathione, as these animals also show a decrease in the activity of glutathione peroxidase and an increase in the degree of lipid peroxidation [134]. In fact, MDMA administration to mice maintained with a standard diet caused a decrease in glutathione peroxidase and superoxide dismutase activities and an increase in lipid peroxidation in several regions of mouse brain [135]. Finally, transgenic mice overexpressing CuZn superoxide dismutase have been shown

to be resistant to the neurotoxic action of MDMA [136]. A recent study by Fornai and coinvestigators [137] reported DNA oxidation and DNA single-strand breaks accompanied by increased clustering of heat shock protein-70 (HSP-70) in the nucleus close to chromatin filaments in the substantia nigra and striatum of mice seven days following repeated MDMA administration. The presence of ubiquitin-positive inclusion bodies has also been reported. These findings suggest that MDMA-induced oxidation involves neuronal cell bodies and extends to the nucleus of neurons.

The source of free-radical formation is controversial. There are data supporting a role for extracellular dopamine in producing free radicals and neurotoxic damage. However, other data fail to support this contention. According to the first hypothesis, dopamine, released massively after MDMA administration, is transported into the 5-HT-depleted terminals and deaminated by monoamine oxidase type B (MAO_B), resulting in the production of hydrogen peroxide and hydroxyl free radicals [128, 138]. This hypothesis fails to demonstrate why MDMA produces damage in brain areas with little dopaminergic innervation such as the hippocampus, where the reduction in several serotonergic parameters is even more pronounced and persistent than that detected in striatum. In addition, there are data indicating that administration of dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA), while enhancing the MDMA-induced dopamine release in the striatal dialysate, nevertheless fails to alter the subsequent neurodegeneration in this region [31].

Free-radical formation is increased by the acute hyperthermia produced by MDMA administration, and the rise in 2,3-DHBA levels in cerebral dialysate was markedly inhibited when the hyperthermic response was prevented. This result provides a plausible explanation as to why hypothermia or normothermia is neuroprotective against MDMA-induced damage and why hypothermia is neuroprotective against other forms of neurodegeneration such as that produced by transitory cerebral ischemia [139, 140]. However, although hyperthermia markedly enhances free-radical production, neurodegeneration can occur in normothermic animals [47], so hyperthermia is not an absolute requirement for the expression of the neurotoxic damage.

The 5-HT nerve endings are the site of the enhanced free-radical formation since a prior 5-HT lesion (produced by pretreatment with fenfluramine) prevented the MDMA-induced rise in free-radical formation [77]. This proposal was supported by the subsequent study by Shankaran and coinvestigators [141], who found that fluoxetine attenuated the MDMA-induced free-radical production. This indicates that free-radical production occurs following the transport of MDMA or a neurotoxic metabolite into the 5-HT nerve terminal by activation of the 5-HT transporter.

17.1.4.3.3 Hyperthermia. In addition to being an immediate risk factor of exposure to MDMA, the drug-induced hyperthermia plays an important role in the development of neurotoxicity in experimental animals, and numerous studies have shown that the hyperthermic reaction following MDMA, although not essential, does modulate the degree of long-term damage. Thus, a close correlation has been found between the hyperthermic response and the degree of neurotoxicity in rats [48]. Animals maintained at an elevated room temperature (26–33°C) have an augmented hyperthermic response and show a greater degree of neurotoxicity [107, 142, 143]. In contrast, physical and pharmacological modifications that either attenuate or prevent the hyperthermic reaction induced by the drug attenuate or prevent the

neurotoxic effect. Thus, the administration of MDMA to rats maintained at low ambient temperature (10°C) prevents the hyperthermic response and either attenuates or eliminates the long-term serotonergic toxicity [142, 144].

A similar observation has been made in the mouse where administration of the drug to mice at an ambient temperature of 15°C reduced body temperature by approximately 2°C and blocked the long-term dopaminergic damage [145]. Furthermore, restraint, which protects mice from the neurotoxic effects of MDMA, is thought to mediate its effect by lowering body temperature [146].

Further evidence for the importance of the hyperthermic response in the degree of neurotoxicity derives from neuroprotection studies. A number of compounds, including haloperidol, pentobarbital, reserpine, α -methyl dopamine, dizocilpine, and ketanserin, have been shown to be protective against the neurotoxic effects of MDMA in the rat because they prevent the hyperthermic response to the drug or produce hypothermia [31, 46, 143, 147, 148]. The protective effect of these compounds disappears when the temperature of the animals is kept similar to that in animals treated with just MDMA. Clomethiazole appears to possess a partial protective action which is independent of its temperature-lowering effects [149].

Despite the preceding evidence on the correlation between the magnitude of the acute hyperthermia and the degree of neurotoxic damage, MDMA may also cause neurotoxic damage in the absence of a hyperthermic response. The repeated administration of low-dose MDMA (4 mg/kg twice daily for four days) produced a substantial neurotoxic effect in Dark Agouti rats but caused only a slight increase in temperature above saline-treated controls after the first dose. This increase in temperature after the initial dose was not sufficient in itself to produce a neurotoxic effect [47]. In addition, high doses of MDMA have also been reported to produce serotonergic toxicity in the absence of a hyperthermic response [142].

Taken together, this evidence indicates that hyperthermia has an important modulatory role but is not an essential factor in the neurotoxicity induced by the drug. Other factors such as high doses or increased frequency of dosing are also important factors and may overcome a lack of hyperthermic response to produce neurotoxicity.

17.1.4.3.4 Monoaminergic Transporter. Evidence indicating a role of the 5-HT transporter in the long-term damage on 5-HT nerve terminals induced by MDMA is based on the findings that 5-HT transporter inhibitors prevent the long-lasting loss of 5-HT brain concentration. The first study showing the neuroprotective effect of fluoxetine against MDMA neurotoxicity is the report of Schmidt [71]. This study shows that coadministration of this selective 5-HT uptake inhibitor completely blocked the reduction in cortical 5-HT concentrations one week after MDMA. Administration of fluoxetine at various times after MDMA revealed that the long-term effects of the drug could be partially blocked by the uptake inhibitor as long as 6 h after drug administration. The neuroprotective effect of fluoxetine administered concurrently with MDMA was confirmed subsequently [46, 82, 141] and extended to fluvoxamine [82] (Fig. 17.2). Neither fluoxetine nor fluvoxamine altered MDMA-induced acute hyperthermic response [46, 82]. Fluoxetine continued to provide total protection when given up to four days before MDMA (Fig. 17.2), but fluvoxamine only produced neuroprotection when coadministered with MDMA (Fig. 17.2). This longer-lasting neuroprotective effect of fluoxetine might be due to the persistent

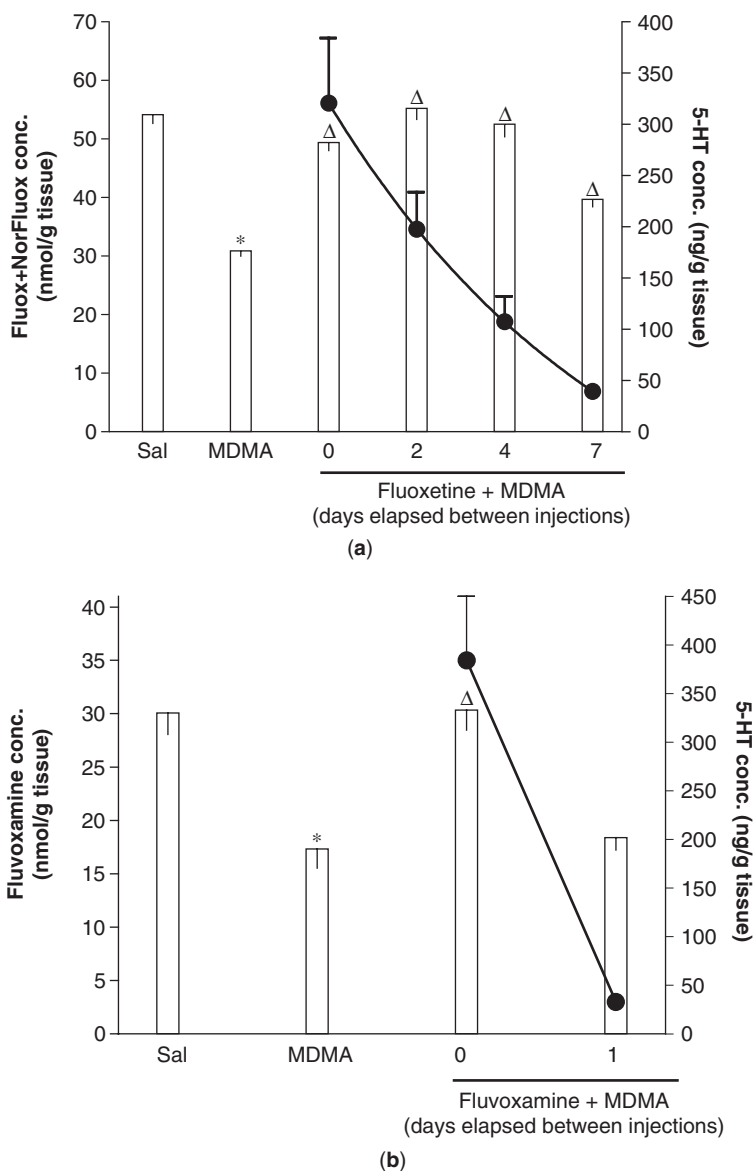


Figure 17.2 Effect of (a) fluoxetine and (b) fluvoxamine on MDMA-induced decrease in cortical 5-HT concentration 1 week after injection. Two injections of fluoxetine (10 mg/kg, i.p.) with an interval of 60 min were given 2, 4, or 7 days before MDMA (15 mg/kg, i.p.). Two injections of fluvoxamine (15 mg/kg, i.p.) with an interval of 60 min were given 1 day before MDMA. A group of animals received fluoxetine or fluvoxamine 5 min before and 55 min after MDMA. Results are shown as mean \pm standard error of the mean (SEM) ($n = 6-16$). Different from saline: (*) $p < 0.001$. Different from MDMA: (Δ) $p < 0.001$. Curves show the corresponding time course of fluoxetine plus norfluoxetine (a) and fluvoxamine (b) concentrations in cortex after administration of two doses of fluoxetine (10 mg/kg, i.p.) or fluvoxamine (15 mg/kg, i.p.) with an interval of 60 min. Levels were measured at 0.5 h and 2, 4, and 7 days after the last dose of fluoxetine and at 0.5 h and 1 day after the last dose of fluvoxamine. Results are shown as mean \pm SEM ($n = 5$ at each time point).

presence of fluoxetine and its main active metabolite norfluoxetine in the brain (Fig. 17.2). Both compounds inhibit the 5-HT transporter and could be blocking the entry of a toxic metabolite of MDMA into 5-HT nerve terminal [82]. In contrast to fluoxetine, fluvoxamine generates several metabolites, none of which appears to be pharmacologically active. Moreover, the parent compound undergoes a rapid plasma clearance in such a way that fluvoxamine brain concentrations are practically undetectable 24 h after injection (Fig. 17.2).

It has been reported that fluoxetine not only prevents MDMA-induced neurotoxicity but also inhibits hydroxyl radical formation [141]. Fluoxetine administration 1 h prior to or 4 h following MDMA administration reduced the MDMA-induced formation of 2,3-DHBA and also attenuated the MDMA-induced depletion of 5-HT in the striatum. As fluoxetine does not modify the *in vitro* generation of 2,3-DHBA, authors indicate that a potential mechanism by which fluoxetine attenuates the MDMA-induced formation of hydroxyl radicals is by preventing the entry into the 5-HT terminal of reactive substances that are capable of generating free radicals.

Not only is the long-term depletion of 5-HT dependent on the activation of the 5-HT transporter but, since fluoxetine is able to attenuate the immediate 5-HT release induced by MDMA in both the striatum [27] and hippocampus [23], it appears that acute MDMA-induced 5-HT release also involves a carrier-mediated mechanism.

Monoaminergic transporters also appear to be involved in MDMA-induced neurotoxicity in mice. MDMA induces a long-lasting depletion of dopamine concentration in the striatal neurons of the mouse, and administration of the dopamine uptake inhibitor GBR 12909 provides substantial protection [150]. Using *in vivo* microdialysis it has been shown that N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride monohydrate (GBR 12909) inhibited the MDMA-induced increase in free-radical formation in the striatum and enhanced the acute release of dopamine induced by MDMA [130]. Altogether these data suggest that, in mice, (1) dopamine release does not involve a transporter-mediated mechanism, (2) MDMA probably enters into the dopamine nerve terminal by passive diffusion, (3) free-radical formation is not associated with dopamine release, and (4) free-radical-producing neurotoxic metabolites may enter the dopamine nerve ending via the dopamine uptake site.

17.1.4.3.5 Cytokines and Microglia. It is well established that cytokines such as interleukin-1 β , interleukin-6, and tumor necrosis factor- α increase body temperature by acting through direct or indirect mechanisms on the brain. In particular, interleukin-1 β has been shown to be involved in the development of the hyperthermic response induced by exogenous pyrogens such as lipopolysaccharide [151], turpentine [152], and leptin [153]. Administration of interleukin-1 β antibodies or an interleukin-1 receptor antagonist to experimental animals inhibits the rise in temperature induced by these external inflammatory stimuli.

Recently, it has been shown that immediately following MDMA administration to rats there is an acute and dramatic increase in interleukin-1 β concentration in the hypothalamus that appears at an early time point after MDMA administration and is of short duration, levels returning to basal values 12 h after drug injection [154]. A similar but less pronounced effect was observed in cortex. It is worth mentioning that under physiological conditions the hypothalamus produces significantly greater amounts of pro-interleukin-1 β than frontal cortex and that this precursor is

converted to its bioactive form and released in a more efficient way in the hypothalamus than in frontal cortex. Thus, pro-interleukin-1 β immunoreactivity, caspase-1-like protease activity, and interleukin-1 β levels are higher in the hypothalamus than in the frontal cortex [154, 155].

Interestingly, there was a clear dissociation in the time course of the changes induced by MDMA on body temperature and interleukin-1 β release. While a marked hyperthermia was evident within the first 20 min of MDMA administration [44], with rectal temperature peaking 60 min after treatment and remaining elevated for over 12 h, the increase in levels of interleukin-1 β peaked at 3 h [154]. These data, and the more definitive observation that intracerebroventricular administration of interleukin-1 receptor antibody did not modify the peak hyperthermic response immediately following MDMA, indicate that interleukin-1 β production could be a consequence, rather than the cause, of hyperthermia and that hyperthermia could represent a signal generated by MDMA which occurs early enough to allow secretion of interleukin-1 β and probably a host of other soluble factors from the microglia. In line with these results, when animals are kept at an ambient temperature of 4°C during MDMA treatment, the hyperthermic response is totally abolished and there is a significant reduction in interleukin-1 β production. These data all indicate that release of interleukin-1 β is, in part, a consequence of the hyperthermia [154].

The rise in interleukin-1 β release following MDMA is accompanied by an enhancement of pro-interleukin-1 β production and/or an increase in caspase-1-like protease activity, the enzyme required for maturation of interleukin-1 β , in the frontal cortex but not in the hypothalamus [155]. Interleukin-1 β is generated as an inactive 31-kDa precursor protein (pro-interleukin-1 β) [156] which is proteolytically processed into the 17-kDa mature interleukin-1 β by a specific intracellular cysteine protease, the interleukin-1 β converting enzyme (ICE), also termed caspase-1 [156]. MDMA increased the immunoreactivity of pro-interleukin-1 β in frontal cortex, not in hypothalamus, 3 and 6 h after administration. Caspase-1-like protease activity was increased in frontal cortex 3 h after MDMA injection compared with saline-treated animals. No change in caspase-1-like protease activity was observed in hypothalamus. Altogether these data indicate that MDMA alters, in a region-specific manner, the mechanisms which regulate interleukin-1 β production in the brain of Dark Agouti rats and suggest that the release of interleukin-1 β in hypothalamus may be regulated independently of caspase-1 activation.

In addition to increasing interleukin-1 β release, MDMA also induced an increase in the density of peripheral benzodiazepine receptor binding sites, labeled with [3 H]PK 11195 [(1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)3-isoquinolinecarboxamide)], in both the hypothalamus and cortex of the rat. This increase could be reflecting an activation of microglia as revealed by immunohistochemical studies in the anterior hypothalamus. However, and in contrast to the increase in interleukin-1 β release, neither the upregulation of peripheral benzodiazepine receptor binding sites nor the staining for OX-42 (monoclonal antibody directed toward complement receptor 3), which stains activated microglia, was significantly modified when the hyperthermic response to MDMA was abolished. These results, together with the fact that the maximal microglial activation occurs 24 h after MDMA (when the hyperthermia induced by MDMA has disappeared), indicate that a hyperthermic response may not be necessary for the morphological changes that occur in the microglia.

17.1.4.4 *Functional Consequences of Neurotoxic Lesion.*

17.1.4.4.1 *On Thermoregulation.* While an MDMA-induced neurotoxic lesion does not alter the hyperthermic temperature response of rats to a further challenge dose of MDMA when the animals are housed in normal room temperature conditions (20°C), it does modify the response in rats housed in a warm (30°C) room. In such conditions, the hyperthermic response is prolonged compared to nonlesioned animals [50]. This prolongation appears to be due to the MDMA-induced loss of cerebral 5-HT since a prolonged response is also seen in rats pretreated with the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) or either of the 5-HT antagonists methysergide and WAY 100635 [157]. The data suggest that a decrease in 5-HT function impairs heat loss in rats housed in warm conditions and is supported by the fact that PCPA pretreatment increases heat-induced mortality in rats housed at high ambient temperature [158, 159].

Other evidence indicates an MDMA-induced neurotoxic lesion can impair heat loss. When lesioned rats are placed in a warm environment and then returned to normal temperature conditions, their rectal temperature remains elevated for longer than control rats [49, 160]. Again normal heat loss mechanisms appear to have been impaired by the neurotoxic lesion. The experiment with WAY 100635 indicates the involvement of the 5-HT_{1A} receptor and it can be proposed that the lesion impairs 5-HT release onto 5-HT_{1A} receptors, thereby producing this defect in thermoregulation. No clinical studies have been performed, but the results suggest that heavy users of MDMA should exercise caution in using the drug in hot room conditions in order to avoid possible problems with hyperthermia.

17.1.4.4.2 *On Behavior.* Most experimental studies on MDMA have mainly focused on the behavioral changes associated with the immediate neurochemical effects induced by MDMA. There is relatively little information on the consequences of the long-lasting depletion of brain 5-HT induced by MDMA on behavioral and cognitive functions.

Marston and coinvestigators [161] performed a study to explore the posttreatment consequences of a 3-day neurotoxic exposure to MDMA in the rat using a variety of behavioral paradigms. Male Lister Hooded rats were injected twice daily with ascending doses of MDMA (10, 15, and 20 mg/kg) over 3 days and behavior was analyzed over the following 16 days. Three weeks after the period of exposure to MDMA, rats showed a persistent depletion of 5-HT (40–60%) in cortex, hippocampus, and striatum and no change in dopamine levels. MDMA exposure resulted in a lasting cognitive impairment as it was found that the MDMA-treated group did not show the progressive improvement in performance of the delayed nonmatched-to-place procedure seen in the control group. Delay-dependent impairments in accuracy are often attributed to perturbations in short-term memory and related systems. The level of locomotor activity of the MDMA-treated rats was significantly greater than the saline controls during the MDMA dosing period and 3 days later but thereafter was indistinguishable from control values. Different results have been reported by Wallace and coinvestigators [162] following administration of 10 mg/kg of MDMA four times 2 h apart to Sprague–Dawley rats and subsequent testing of diurnal and nocturnal spontaneous locomotor activity. Two weeks after dosing rats showed a depletion of striatal 5-HT content of approximately 50% and also exhibited significant reductions in both diurnal and nocturnal locomotor

activity. This effect may be due to the reported regulatory actions of 5-HT on the sleep–wake cycle [163, 164]. In fact, rats with a significant reduction in the neocortical density of 5-HT transporter 3 weeks after exposure to a single 15-mg/kg dose of MDMA show long-term changes in regulation of circadian rhythms and sleep generation [165].

Prior MDMA exposure also alters anxiety-related behaviors, but results have been conflicting and may be dependent on the basal anxiety level of the rat strain being investigated [166]. Using the same paradigm for behavioral testing (elevated-plus maze), obtaining a similar decrease in cerebral 5-HT concentration (30–40% loss), and considering a similar delay for testing animals following MDMA administration (70–90 days posttreatment), Morley and coinvestigators [167] reported anxiety-like behaviors in Wistar rats while Mehan and coinvestigators [168] described an apparent anxiolytic response in Dark Agouti rats. It thus appears that a 5-HT lesion may produce an anxiolytic response in an anxious strain (Dark Agouti) but an anxiogenic response in strains displaying low endogenous anxiety (Wistar rats) [166].

Acute exposure of young Lister Hooded rats to MDMA produces a long-term anxiety-like behavioral response in the social interaction paradigm, even though there were only modest reductions in indole brain concentrations and no change in the density of cortical 5-HT transporters [167]. Similar results were obtained following exposure of young Wistar rats to low and repeated doses of MDMA, the anxiety-related behavior being accompanied by changes in 5-HT_{2A} receptor function [169].

Recently McGregor's team [170] has performed a study to investigate the long-lasting behavioral and neurochemical effects of combined MDMA and methamphetamine administration in the rat. Animals received four injections, one every 2 h, of MDMA (2.5 or 5 mg/kg per injection) or methamphetamine (2.5 or 5 mg/kg per injection) given alone or in combined low doses (1.25 + 1.25 mg/kg per injection or 2 + 2 mg/kg per injection). Several weeks after drug administration, both MDMA + methamphetamine groups, both metamphetamine groups, and the higher dose MDMA group showed decreased social interaction relative to controls while both MDMA + methamphetamine groups and the lower dose MDMA group showed increased anxiety-like behavior on the emergence test. MDMA treatment caused 5-HT and 5-HIAA depletion in several brain regions, while methamphetamine treatment reduced dopamine in the prefrontal cortex. Combined MDMA + methamphetamine treatment caused 5-HT and 5-HIAA depletion in several brain regions and a unique depletion of dopamine and DOPAC in the striatum. These data have potentially important implications for public awareness as they demonstrate specific adverse behavioral and neurochemical effects of MDMA and methamphetamine given alone at relatively low doses in a novel one-day regimen. Results also show that coadministration of MDMA and methamphetamine leads to adverse effects that may be more pronounced and consistent than those observed with similar doses of MDMA or methamphetamine administered alone.

As mentioned above, the study of Marston and coinvestigators [161] was the first to report that administration of ascending doses of MDMA for three days resulted in an impaired cognitive behavior and a pronounced depletion of 5-HT brain concentration. Subsequently, Broening and coinvestigators [171] reported that exposure to MDMA in rats during stages analogous to early and late third-trimester human fetal brain development induces specific types of long-term learning and memory impairments. MDMA exposure on post natal days P11–P20 resulted in dose-related

impairments of sequential learning and spatial learning and memory. These learning deficits reflect a developmentally specific vulnerability in that they were selective, affecting only those animals treated on P11–P20 and not those treated on P1–P10. The effects were also long term in that they were seen in the offspring as adults and not related to any long-term changes in 5-HT, dopamine, or noradrenaline.

An impairment of nonspatial working memory has also been reported in rats exposed to intermittent administration of MDMA every fifth day from P35 to P60 trying to model the typical intermittent MDMA human use pattern in adolescent Sprague–Dawley rats. Five days after dosing animals showed a significant reduction of about 25% in the density of 5-HT transporters in cerebral cortex, not in hippocampus [172].

In nonhuman primates there have been few studies performed to evaluate cognitive functions such as learning and memory. Frederick and coinvestigators [173–175] did not find any long-term alteration in performance on a number of operant tasks in rhesus monkeys exposed to neurotoxic doses of MDMA. Thus, repeated exposure to doses of MDMA sufficient to produce long-lasting changes in brain neurotransmitter systems results in residual effects (e.g., tolerance, sensitivity) on behavioral task performance when subjects are subsequently challenged with acute MDMA, whereas baseline (nonchallenged) performance of these tasks after such exposure generally remains unchanged. Similarly, Taffe and coinvestigators [176] found no apparent lasting effect induced by MDMA on a battery of cognitive/behavioral measures in rhesus monkeys with cerebrospinal fluid (CSF) 5-HIAA concentration reduced by 40–50%. Winsauer and coinvestigators [177] showed that neurotoxic doses of MDMA failed to disrupt learning in squirrel monkeys in spite of impairment being demonstrated following administration of fenfluramine. Nevertheless, although cognitive/behavioral measures performed in nonhuman primates following neurotoxic doses of MDMA are normal, brain stem auditory-evoked potentials are abnormal for a period of at least three months after MDMA dosing [176]. This long-lasting effect is consistent with a loss of 5-HT innervation of brain stem auditory nuclei and suggests that auditory pathways are much more sensitive to the deleterious effect of MDMA. It is interesting to note that studies which have failed to show memory impairment in human beings employed nonauditory tasks [178] while those reporting memory disruption showed some auditory memory task [179, 180].

17.1.5 Biochemical and Functional Changes in Human Brain

Neuroimaging studies performed on recreational users of MDMA have shown sustained reductions in brain 5-HT transporter density [181–183] which positively correlated with the extent of previous MDMA use [183] and duration of abstinence [182]. The effect might be more pronounced in women and reversible after prolonged abstinence [184, 185]. A reduction in the CSF 5-HIAA concentration has also been observed in recreational users of MDMA, the reduction being greater in females (46%) than in males (20%) [186, 187]. MDMA users show a downregulation of 5-HT_{2A} receptors in the cerebral cortex while in the abstinent MDMA user group there is an upregulation of 5-HT_{2A} receptors. The combined results of this study suggest a compensatory upregulation of postsynaptic 5-HT_{2A} receptors in the occipital cortex of ex-MDMA users due to low synaptic 5-HT levels [188]. MDMA users do not appear to suffer any reduction in nigrostriatal dopamine

neurons. However, subjects regularly using amphetamine in addition to MDMA did display a 20% loss in the density of striatal dopamine transporters [189].

Neuroendocrine tests have shown that abstinent MDMA users exhibit blunted cortisol and prolactin responses to fenfluramine challenge compared to controls, the prolactin response still being observed after one year of abstinence [190]. The presence of a long-lasting impairment of brain serotonergic function in recreational users of MDMA could potentially account for the reported cognitive and mood impairments, particularly when there is a relationship between the extent of lifetime consumption of MDMA and the severity of cognitive deficits [191–193].

Long-term MDMA use impairs performance in a multitude of cognitive abilities (most notably memory, learning, attention, executive function). Deficits in verbal and visual memory have been most frequently observed [180, 185, 193–195]. Users also show impaired verbal learning, are more easily distracted, and are less efficient at focusing attention on complex tasks [196–198].

Cognitive impairments have also been detected in a recent pilot study focused on adolescent MDMA users [199]. It appears that many of the neuropsychological performance problems reported to occur in MDMA users are not reversed by prolonged abstinence, suggesting the existence of a selective neurotoxic lesion [200]. It is essential to mention that many subjects are polydrug users; therefore, it cannot be stated unequivocally that any effect seen results solely from MDMA use. Cannabis users, whether or not they also used MDMA, showed significantly impaired memory function on word free recall and on immediate and delayed story recall compared to nonusers. The findings highlight the importance of controlling other drug use (particularly cannabis) when investigating persistent effects of MDMA in humans [201]. Gouzoulis-Mayfrank and coinvestigators [202] performed a comprehensive cognitive test battery on 28 recreational Ecstasy users with concomitant use of cannabis only and two equal-size matched groups of cannabis users and nonusers. The test battery included tests of attention, memory and learning, frontal lobe function, and general intelligence. Ecstasy users performed worse than one or both control groups in the more complex tests of attention, in memory and learning tasks, and in the tasks reflecting aspects of general intelligence. Poorer performance scores or longer reaction times in the working memory, verbal memory, and divided attention tasks were associated with heavier Ecstasy and heavier cannabis use. These results raise the concern that Ecstasy use, even in typical moderate recreational doses and possibly in conjunction with cannabis use, may lead to a subclinical cognitive decline in otherwise healthy young people.

Alternatively the problem may relate to the combination of MDMA with another recreational compound; a significant percentage of Ecstasy tablets contain psychoactive compounds other than MDMA. Therefore, although only MDMA and other amphetamine derivatives have been clearly demonstrated to produce neurotoxicity, the possibility cannot be ruled out that neurotoxicity is due to a combination of MDMA and other compounds ingested.

17.2 GHB

In recent years, γ -hydroxybutyric acid (GHB) has grown in popularity as a drug of abuse, due mainly to its ability to produce euphoria and promote relaxation,

increase sociability, and produce a heightened sense of sexuality and an increased disinhibition similar to that produced by ethanol [203]. The drug is most commonly referred to on the street as “liquid Ecstasy” but also goes under the names “liquid X,” “liquid E,” “Grievous Bodily Harm” (a play-on-words with the initials), and “Salty Water” due to the slightly salty taste of its preparations. GHB is usually found as a clear, colorless liquid, almost tasteless with a slight salty taste which is easily masked in alcoholic drinks. These characteristics together with its amnesic and hypotonic effects have led to its use in some cases of “date rape.”

The drug was first synthesized in 1960 [204] as an orally active analog of the neurotransmitter γ -aminobutyric acid (GABA), with the capacity to cross the blood–brain barrier [205]. Due to its ability to induce sleep and coma, its potential use as an anesthetic was explored, but its lack of sufficient analgesia and high incidence of vomiting as well as ability to cause convulsions limited its use.

The drug was first introduced into the North American market in the early 1990s as a dietary supplement available in health food shops for the treatment of anxiety and insomnia and to increase body mass in athletes and body builders. There have been reports of the therapeutic potential of GHB in the treatment of alcohol and opioid withdrawal, but such studies are far from conclusive [206–208].

Reports of its adverse effects followed soon after the introduction of GHB into the market [209], and this led to its prohibition, by the Food and Drug Administration (FDA), for use in humans except under medical prescription. In March 2000, the FDA reclassified GHB as a Schedule I controlled substance. In 2002, the FDA approved the use of GHB in patients suffering from narcolepsy with cataplexy. For this condition only, Xyrem was included in Schedule III of the Controlled Substances Act for medicinal use. Meanwhile the illegal use of GHB remains under the control of Schedule I [210].

GHB is a short-chain fatty acid found in mammalian brain [211] and was first identified in human brain in 1963 [212]. GHB is synthesized during the metabolism of the neurotransmitter GABA via an intermediate product, succinic semialdehyde, by means of the enzyme succinic semialdehyde reductase [213]. However, controversy exists as to whether GABA is the principal endogenous source of GHB. A marked reduction in GABA levels in the brain due to the administration of a glutamate decarboxylase inhibitor does not modify GHB levels [214]. In addition, GHB is found in peripheral tissues such as heart, kidney, and muscle at a higher concentration than that of GABA, suggesting an extraneuronal origin [215, 216]. It has been suggested that another compound in the brain, 1,4-butanediol, could also give rise to GHB by the action of alcohol dehydrogenase and aldehyde dehydrogenase [214, 217].

GHB is rapidly absorbed by oral administration [218], and its effects are evident after 15 min with peak plasma levels being reached between 30 and 60 min after consumption, depending on the dose [205, 219]. It does not bind significantly to plasma proteins [219]. Within the therapeutic range, GHB follows nonlinear pharmacokinetics. At low doses of 12.5 mg/kg the half-life is estimated at 20 min whereas higher doses exhibit longer half-lives. It is mainly eliminated by metabolism to carbon dioxide with only 2–5% being eliminated in urine [220].

17.2.1 Pharmacological Effects

GHB exhibits a steep dose–response curve leading to the appearance of adverse effects with a small increase in dose. The principal effects are those relating to the

central nervous system (CNS) and cardiovascular and respiratory systems; GHB does not appear to affect the hepatic or renal systems [220]. For the most part, the adverse effects are acute in nature, appearing in the first 15 min and disappearing after 7 h.

Adverse effects on the CNS constitute the majority of GHB acute toxic reaction reports. They appear even at intermediate doses (25–50 mg/kg) and consist of sleepiness, dizziness, vertigo, and headaches [218, 219]. At higher doses it can cause the rapid onset of a state of coma (with no participation of the reticular activating system) which is short-lived and apparently fully reversible [221].

With regard to the cardiovascular system, GHB produces bradycardia both as an anesthesia inducer as well as in overdose with cardiac rhythms of fewer than 55 beats per minute having been recorded. Hypotension often accompanies the reduced heart rate.

Respiratory depression, difficulty breathing, and apnea have all been reported following GHB consumption [209, 220].

All the effects listed above are enhanced by ethanol such that the ingestion of both drugs together produces greater toxic effects than those produced by each drug taken alone [222]. In fact, 64% of the emergency room visits due to GHB involve the use of ethanol as well [223].

GHB also produces effects on the visual system, such as myopia and pupils unreactive to light, and the gastrointestinal system, such as nausea and vomiting, as well as motor effects such as clonic movements and uncontrollable shaking and slight hypothermia [209, 220, 224].

On occasion GHB has been associated with psychopathological symptoms such as hostile behavior, belligerence, and agitation. Other psychiatric symptoms such as delirium, paranoia, depression, and hallucinations have also been described [225].

Studies in humans, reports from emergency room admittances, as well as surveys and statements, all point toward a withdrawal syndrome of the characteristics produced by ethanol or benzodiazepine withdrawal. The syndrome appears 1–6 h after the last dose [226] and can last up to 15 days. Initial symptoms include insomnia, anxiety, agitation, tremors, nausea, and vomiting. This is followed by autonomic nervous system instability which manifests itself with diaphoresis, hypertension, shaking, and tachycardia. Following withdrawal after chronic or high-dose use, psychotic symptoms, hallucinations, and delirium have been described [227]. A prolonged abstinence syndrome period has been described which lasts between three and six months and is characterized by dysphoria, anxiety, memory problems, and insomnia. During this time the risk of relapse or development of ethanol or benzodiazepine dependence is high [228].

17.2.2 Mechanism of Action

The mechanism of action of GHB has not been completely elucidated. Although it was first synthesized as a GABA analog, early experimental evidence showed it to have different biochemical and behavioral effects.

There is a large body of evidence indicating that GHB produces many of its effects by means of interaction with the GABAergic system. GHB has no affinity for the GABA_A receptor [229] but acts as a partial agonist at the GABA_B receptor [230], although at concentrations higher than those normally found in the brain. In

GABA_B (–/–) knockout mice, GHB does not produce hyperlocomotion, hypothermia, increases in dopamine synthesis, or increases in delta waves as measured by EEG, effects which are observed in wild-type mice [231]. Therefore, it appears that at least some of the effects produced by GHB are mediated by the GABA_B receptor.

However, GHB does not consistently exhibit a GABA agonist profile, suggesting that it must act, at least in part, through some other mechanism. This naturally occurring compound has been proposed as a neurotransmitter or neuromodulator in its own right. GHB is found in high concentrations in the substantia nigra and hypothalamus [215, 232]. It is released from neurons in a calcium-dependent manner in response to potassium, and these neurons contain highly specific sodium-dependent uptake sites [233, 234]. In addition, G-protein-coupled GHB receptors have been located in dopaminergic areas on GABAergic and/or enkephalinergic neurons [235].

Regardless of the exact mechanism of action, exogenous administration of GHB has been shown to modulate various neurotransmitter systems, including the dopaminergic system. GHB inhibits the release of dopamine [236], and animals show sedation and a dose-dependent reduction in locomotor activity [231, 237]. The reduction in dopamine is inhibited by GABA_B antagonists [238]. At high doses the rapid reduction in dopamine release is followed by an accumulation of tissue dopamine in the frontal cortex [239], most likely due to an increase in the activity of tyrosine hydroxylase, and an increase in its release in the striatum and various areas of the corticolimbic system [240].

GHB does not bind to opioid receptors [241], although some of its effects can be reproduced by opioid agonists. Local administration of GHB produces an increase in the release of endogenous opioids which appears to be mediated by the decrease in dopaminergic function [241].

GHB increases 5-HT turnover [242] and synthesis through a modulatory effect on GABA release [213].

17.2.3 GHB and Addiction

In drug discrimination studies in rats, GHB has been shown to be partially substituted for by morphine, lysergic acid diethylamide (LSD), *d*-amphetamine, GABA agonists (baclofen), and chlordiazepoxide [243]. These effects appear to be mediated, in part, by the GABA system since 3-Aminopropyl (diethoxymethyl) phosphinic acid (CGP35348), a GABA_B receptor antagonist, blocked discrimination both at low and especially at high doses of GHB [244]. In addition, GHB has been shown to substitute for intermediate doses of ethanol [245].

In reinforcement studies, after repeated GHB administration, animals showed a place preference suggesting that the reinforcing effects of the drug are weaker than those produced by cocaine or opiates, which require only single exposure [246].

Self-administration studies indicate that rats trained to drink GHB prefer GHB in a subsequent two-drinking-bottle paradigm [247]. Furthermore, rats receiving GHB in response to nose-pokes will respond with more nose-pokes than those animals who are yoked to passively receive the same dose of GHB and who receive a vehicle in response to nose-pokes [248].

There is some evidence of tolerance to the motor dysfunction effects in mice and there is cross-tolerance with ethanol [249]. A withdrawal syndrome has been

described using the same scale as is used to evaluate ethanol intoxication withdrawal following repeated daily administration for three to six days [250].

17.3 KETAMINE

Another popular club drug is ketamine, a phencyclidine derivative, first introduced into clinical practice in the 1960s as a dissociative anesthetic [251]. Its clinical use has diminished, but it is still used in circumstances where its dissociative/analgesic effects are advantageous, such as in patients with burns. It is administered by the intravenous or intramuscular routes, and drugs such as diazepam are often coadministered in order to reduce the incidence of hallucinations and/or psychosis symptoms. These symptoms appear to be less common in children. Ketamine is still quite widely used in veterinary practice for the sedation of animals for surgery, transport, or euthanasia [252].

It is thought that ketamine first entered the club scene as an adulterant of MDMA (Ecstasy), but it is now consumed in its own right [253]. On the street it is known by a number of names, including “K,” “Special K,” “Vitamin K,” or “Kit-kat.” Supply of ketamine originates mainly from the diversion of prescription products. The drug can be injected either intravenously or intramuscularly [254], taken orally either as a powder or liquid, snorted as a powder, or smoked [255]. Orally it is less well absorbed and undergoes extensive first-pass metabolism [254]. The drug is tasteless, odorless, and colorless, which makes it suitable for its secret addition to drinks in order to facilitate sexual assault. This property, as well as its ability to cause anterograde amnesia and hallucinations making the victims unreliable as witnesses, has led to its use in cases of date rape [256].

17.3.1 Pharmacological Effects

Ketamine has a rapid onset of action, lasting approximately 30–45 min. Low doses produce a dreamlike dissociative effect with analgesia and can lead to involuntary lesions. Higher doses can lead to hallucinations and vivid dreams as well as to amnesia. Users describe an “out-of-body” experience where the mind is taken to “K-land” or “K-hole,” an experience that can be either spiritual or unpleasant depending on the individual [257]. Neurological toxicity may manifest itself as nystagmus, mydriasis, agitation, slurred speech, confusion, delirium, floating sensation, hypertonus, rigidity, anxiety, vivid dreams, hallucinations, hostility, and seizures [255]. Cardiovascular toxicity includes hypertension, tachycardia, and palpitations, and respiratory toxicity may appear in the form of depression and apnea [252]. Rhabdomyolysis has also been reported [258]. Its effects can be potentiated by ethanol, GHB, or benzodiazepine ingestion.

Ketamine is an *N*-methyl-D-aspartate (NMDA) agonist that also acts at non-NMDA–glutamate receptors, nicotinic and muscarinic cholinergic receptors, σ receptors, monoaminergic receptors, opioid receptors, and sodium and calcium channels. At the NMDA channels, ketamine binds noncompetitively to the phencyclidine site and inhibits glutamate activation of the channel. The drug also stimulates nitric oxide synthesis and acts as an inhibitor of norepinephrine, dopamine, and 5-HT uptake [251, 259, 260]. In addition, it produces dopamine release in the nucleus accumbens [261],

and there are reports of the induction of dependence in rats [262]. Therefore, it appears that ketamine has the potential to be an addictive drug of abuse.

17.4 FLUNITRAZEPAM (ROHYPNOL)

Flunitrazepam is a fast-acting potent hypnotic/sedative benzodiazepine which, although never approved for use in the United States, has been widely used in Europe and some Latin American countries under the brand name Rohypnol for the treatment of insomnia, sedation, and preanesthesia [252]. The drug has become increasingly popular among teenagers and young adults and is often consumed in combination with alcohol and/or other illicit drugs. Reports suggest that it is the preferred benzodiazepine of heroin addicts and has also been identified in numerous cases of drug-facilitated sexual assault due to its induction of anterograde amnesia.

The drug is generally taken orally as the commercially available pill or dissolved in a drink. The drug has good bioavailability, is rapidly distributed from plasma into tissue, and is metabolized by the liver into two active compounds [263]. The effects appear after approximately 20–30 min and last 4–6 h, although residual effects can be observed at much later time points. The half-life of the drug is approximately 20 h and the metabolites are excreted renally.

17.4.1 Pharmacological Effects

As with other benzodiazepines, flunitrazepam produces a reduction in anxiety, muscle relaxation, and sedation. In addition, the drug produces amnesia which is enhanced by the concurrent ingestion of alcohol.

At higher doses, flunitrazepam can cause lack of muscle control and loss of consciousness. The drug can produce hypotension, dizziness, confusion, visual disturbances, urinary retention, and, occasionally, aggression [263].

As with other benzodiazepines, flunitrazepam can cause dependence and a withdrawal syndrome, although due to its long half-life this may take several days to appear and persists for weeks after the last dose. The symptoms include headache, tension, anxiety, restlessness, insomnia, loss of appetite, tremor, and perceptual disturbances.

17.5 LSD

LSD belongs to the hallucinogenic class of drugs. Hallucinogens are defined as pharmacologically active substances which alter consciousness, often in a dramatic and unpredictable manner, and which at high doses can produce delirium, hallucinations, separation from reality, and in some cases death, which is not due to overdose. These substances are also referred to as psychomimetics since they alter cognitive functions and personality and mimic psychosis.

The hallucinogens are included in Schedule I of the Controlled Substances Act and are traditionally classified into one of two groups: phenylalkylamines (mescaline, DOB, DOI) and the indolealkylamines (DMT, 5-MeO-DMT, psilocybin, LSD, harmaline, bufotenine) (Table 17.1).

TABLE 17.1 Hallucinogens: Dose, Duration of Effects, and Route of Administration in Humans

Hallucinogen	Dose (mg)	Duration of Effects	Route of Administration
DMT	60–100	1 h	Smoked, inhaled, nasal, injection
5-MeO-DMT	6–20	20–30 min	Smoked, inhaled, nasal, injection
Psilocybin	6–20	4–6 h	Oral
LSD	0.06–20	10–12 h	Oral
Mescaline	200–400	10–12 h	Oral
DOB	1–3		Oral
DOI	1.5–3		

DMT: *N,N*-dimethyltryptamine; 5-MeO-DMT: 5-methoxy-dimethyltryptamine; DOB: 2,5-dimethoxy-4-bromoamphetamine; DOI: 2,5-dimethoxy-4-iodoamphetamine

LSD is a prototypical hallucinogen which was first introduced to Europe and North America in 1949 [264]. It is a semisynthetic molecule derived from lysergic acid, which is produced by a fungus which grows on rye. Its effects were first identified by a chemist, A. Hoffman, working for Sandoz Pharmaceuticals, who experienced the mental effects of the drug after working with the compound [265]. The drug, as with most hallucinogens, is orally active and is the most potent of all hallucinogens [266]. At the other end of the spectrum is mescaline, which is the least potent of the classical hallucinogens (Table 17.1).

17.5.1 Pharmacological Effects

The main effects of LSD, which are common to most of the hallucinogens, are listed in Table 17.2. It is generally considered safe from a physiological standpoint since it alters consciousness at low doses which are not toxic to the cardiovascular, renal, or hepatic systems. At lower doses (25–50 µg) the drug produces mild visual effects which are not considered hallucinations or distortions of perception. At higher doses (250 µg) the drug produces sympathetic arousal characterized by increased pulse and blood pressure, dilated pupils causing blurred vision, hyperreflexia, and slight pyrexia [264]. In one reported case of multiple overdose, hemorrhage occurred which may have been mediated by LSD antagonism of platelet 5-HT function [267].

17.5.2 Mechanism of Action

LSD appears to mediate most its hallucinogenic effects by its action at the 5-HT_{2A} receptor. Studies carried out in experimental animals reveal that 5-HT_{2A} receptor antagonists such as ketanserin and pirenperone block the discriminative effects of both classes of hallucinogens in rats trained to discriminate between the effects of saline and the training drug [268, 269]. In addition, other studies with more highly selective antagonists of the 2A receptor over the 2C receptor have confirmed the 5-HT_{2A} receptor as the target [270]. Similar observations have been made in humans

TABLE 17.2 Pharmacological Effects and Adverse Reactions of Hallucinogens

Somatic Alterations	Alterations of Perception	Mental Alterations	Adverse Reactions
Dizziness, weakness, shaking, nausea, sleepiness, paresthesias, blurred vision	Distortion of shapes and colors, difficulty in focusing, heightened sense of hearing, synesthesias (unusual)	Mood changes (happiness, sadness, or irritability), tension, distorted perception of the passage of time, difficulty in expressing thoughts, depersonalization, dream-like state, and visual hallucinations	Anxiety, agitation (bad trips), flashbacks, precipitation of psychosis and depression (in susceptible individuals), suicide

[271, 272]. The development of tolerance, as seen in both animals and humans, appears to be related to the selective downregulation of the 5-HT_{2A} receptor following daily administration [273].

Hallucinogen-induced activation of 5-HT_{2A} receptors located on neocortical pyramidal neurons leads to an increase in glutamate levels in the prefrontal cortex [274] presumably through an action mediated by the presynaptic receptors of thalamic afferents. This release of glutamate is thought to contribute to the behavioral effects produced by the hallucinogens since antagonism of the metabotropic glutamate mGlu2/3 receptor, a putative presynaptic autoreceptor on glutamatergic neurons [275], increases appropriate responding in rats trained to discriminate LSD [276]. Opposite effects were observed with an mGlu2/3 agonist. Agonists and antagonists of the mGlu2/3 receptor have been observed to decrease and increase glutamate release, respectively [277, 278].

Although activation of 5-HT_{2A} receptors is accepted to be an essential component for the acute behavioral effects of the hallucinogens, it is possible that interaction with other receptors in the CNS may modulate their overall psychopharmacological effect. The hallucinogens, with the exception of LSD, have no affinity for dopaminergic receptors or for the dopamine transporter and therefore do not directly alter dopaminergic neurotransmission. However, systemic administration of DOI produces a 5-HT_{2A}-mediated increase in dopamine release in the prefrontal cortex [279], and local infusion of DOI in the nucleus accumbens also increases dopamine release [280]. Therefore it is possible that dopamine may participate in some of the behavioral effects of these drugs. In fact, it is thought that later onset behavioral effects of LSD appear to be mediated by dopamine pathways [281].

17.5.3 Hallucinogens and Addiction

In contrast to other drugs of abuse, the hallucinogens do not produce dependence or addiction and are not considered to be reinforcing substances [282] in spite of producing alterations in dopamine neurotransmission either directly or indirectly

(see above). In fact, in the rhesus monkey LSD produces negative reinforcement [283], although in the rat the drug appears to have weak reinforcing properties [284] which may be related to the drug's direct action on dopamine receptors. This lack of addictive properties is interesting to note in light of the fact that modifications of dopamine neurotransmission, in particular dopamine release in the mesolimbic pathways, seem to be involved in the mechanism of action of drugs of abuse which cause dependence.

Studies designed to train animals to self-administer classical hallucinogens have failed, indicating that these substances do not possess the pharmacological properties necessary to establish and maintain a state of dependency. The drugs are generally consumed sporadically in a noncompulsive manner, and the majority of experimental first-time consumers do not go on to establishing a pattern of regular consumption. This pattern of consumption contrasts with the compulsive nature of amphetamine, cocaine, and opiate abuse which alter the pathways of reward and produce craving.

Tolerance to the behavioral effects of LSD occurs after daily administration for four to seven days and lasts for approximately three days [285]. Cross-tolerance occurs between LSD and other hallucinogens such as psilocybin and mescaline [286, 287].

17.6 SUMMARY

This chapter summarizes the latest literature on MDMA (Ecstasy), the most popular drug used by teens and adults in raves and other dance parties, and other compounds also categorized as club drugs—GHB, ketamine, Rohypnol, and LSD. Consumption, in general, appears to have increased in Western countries over the last 20 years, although trends show a variety of users and settings. Each drug produces diverse characteristic acute behavioral and biochemical effects which require different specific acute-care protocols. However, all of them share the common property of producing rewarding effects probably by activation of the mesolimbic system and the release of dopamine in the nucleus accumbens. MDMA induces long-lasting neurochemical changes reflected by a loss of 5-HT nerve terminals in the brain of rats, guinea pigs, and nonhuman primates. An increasing number of neuroimaging studies indicate that damage might also appear in human beings. The fact that the doses of MDMA that produce damage in experimental animals are similar to that used by humans places consumers in an area of high risk even after relatively short-time exposures to Ecstasy.

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18

MARIJUANA: PHARMACOLOGY AND INTERACTION WITH THE ENDOCANNABINOID SYSTEM

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18.1	Introduction	659
18.2	Endocannabinoid System	662
18.2.1	Cannabinoid Receptors and Signaling	662
18.2.2	Synthesis and Metabolism of Endocannabinoids	663
18.2.3	Development	664
18.3	Marijuana Pharmacology: Implications for Physiology of Endocannabinoid System	665
18.3.1	Pain	666
18.3.2	Cognition	669
18.3.3	Appetite Regulation	670
18.3.4	Neurotoxicity	672
18.3.5	Emesis	673
18.4	Endocannabinoid Role in Reward, Tolerance, and Dependence	675
18.5	Future Directions	676
	Acknowledgments	676
	References	677

18.1 INTRODUCTION

Comprised of the dried leaves of the cannabis plant, marijuana (*Cannabis sativa*) is the most commonly abused illicit substance in the United States today, particularly among adolescents. In 2004, nearly half of seniors in high school had tried marijuana at least once and 20% were current users [1]. Further, while the number of regular adult users was approximately the same during the early part of this decade, the prevalence of marijuana abuse and dependence among these users (especially among racial and ethnic minorities) significantly increased, suggesting a combination of causal factors [2]. Yet, marijuana use is not a new phenomenon. Marijuana and other

constituents of the cannabis plant (e.g., hashish) have a long history of medicinal and religious use which dates back to ancient China. Even in the United States, marijuana was commonly used for medicinal purposes (e.g., nausea, arthritis) and as an intoxicant until after World War I. In the 1930s, however, a concerted effort by the Federal Bureau of Narcotics (a predecessor of the Drug Enforcement Administration) resulted in a change in public attitude toward marijuana. It became to be perceived as a “gateway drug” that led to addiction to “harder drugs” such as heroin. This new perception culminated with the Controlled Substances Act of 1970, in which marijuana was listed as a Schedule I drug with high abuse potential and no accepted medical use. The current debate over medical marijuana highlights this dichotomy between the demonstrated abuse properties of marijuana (as codified by its classification as a Schedule I drug) and growing evidence of its therapeutic potential for a wide variety of medical problems.

Scientific interest in marijuana, although relatively recent, has increased in response to several important discoveries in the field. First, the primary psychoactive constituent of the marijuana plant, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), was isolated and identified [3]. Identification of this substance (Fig. 18.1) allowed further clinical and preclinical research to characterize its pharmacological effects in the body and on behavior. It also allowed manipulation of dosage so that dose dependence of these effects could be evaluated as well as better determination of the psychoactive potency of marijuana. In recent years, the average Δ^9 -THC content of street marijuana in the United States has increased to greater than 4%, although exact concentration in any sample varies considerably depending upon the growing conditions, the plant variety, and the preparation. For example, sinsemilla, the dried flowering tops of unfertilized female plants, may have Δ^9 -THC concentrations as high as 20%. Although it is hypothesized that the effects of the numerous other unique chemical constituents of marijuana (cannabinoids) may modulate the primary effects of Δ^9 -THC, direct

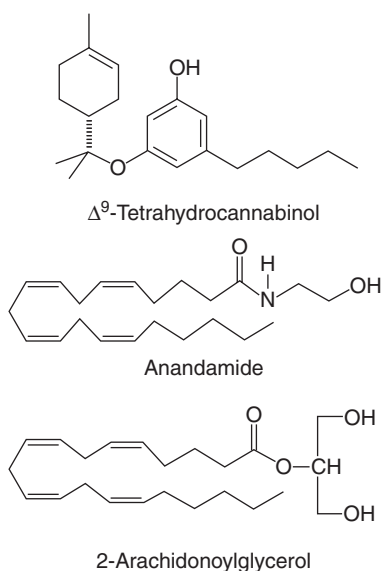


Figure 18.1 Structures of Δ^9 -THC, anandamide, and 2-arachidonylglycerol.

evidence for their role in marijuana's pharmacological effects is lacking. Hence, most of the preclinical mechanistic research on the pharmacological effects of marijuana is actually research on the effects of Δ^9 -THC.

A second discovery that increased scientific interest in marijuana was identification of a cannabinoid receptor (CB₁) in the brain [4]. Prior to this discovery, it was theorized that Δ^9 -THC and other highly lipophilic cannabinoids produced their effects in the central nervous system by disordering neuronal cell membranes and hence interfering with their normal function. Despite the membrane perturbation theory, however, there was ample evidence for a specific action of cannabinoids. Δ^9 -THC is a highly potent drug that produces a distinct profile of pharmacological effects with a strict structure–activity relationship. Each of these factors suggests a selective receptor-mediated action rather than generalized disruption of membranes. In addition, synthetic cannabinoid derivatives were prepared that were extremely potent and enantioselective, thereby enhancing the likelihood of a receptor mechanism. Direct evidence for cannabinoid receptors, however, awaited development of a radiolabeled ligand with which to perform ligand binding studies.

Analysis of binding data with [³H]CP 55,940, the first radiolabeled ligand for CB₁ receptors, revealed a single site that possessed saturable and reversible binding and that displayed selectivity for cannabinoids [4]. Further, the affinity of cannabinoids for this binding site correlated well with their potency in a variety of pharmacological assays, including production of antinociception, catalepsy and hypothermia, and suppression of spontaneous locomotor activity in mice [5]. In addition, a high correlation was found between CB₁ binding affinity and *in vivo* potency in the rat drug discrimination model (an animal model of the subjective effects of psychoactive drugs) and for marijuana-like psychoactivity in humans [6]. Subsequently, a second cannabinoid receptor (CB₂) was discovered in the periphery. Both CB₁ and CB₂ receptors have been cloned [7, 8].

An interest in the endogenous ligand for these cannabinoid receptors sparked the discovery of arachidonoyl ethanolamide (anandamide) in porcine brain [9]. Later, a number of other endogenous cannabinoids (or endocannabinoids) were described, including 2-arachidonoylglycerol (2-AG), 2-arachidonoylglycerol ether (noladin ether), *N*-arachidonoyldopamine, and *O*-arachidonoyl ethanolamine (virodhamine) [see [10] for a review]. The structures of THC, anandamide, and 2-AG are depicted in Figure 18.1. Although the chemical structure of anandamide does not resemble the structure of classical cannabinoids such as Δ^9 -THC or of nonclassical synthetic cannabinoids, such as CP 55,940 [11] and the aminoalkylindoles [12], it shares with these diverse cannabinoids the ability to bind to and activate identified cannabinoid receptors and to produce a similar profile of pharmacological effects, although there are also some differences (see [13] for a review).

Another important tool in cannabinoid pharmacology was development of selective CB₁ and CB₂ cannabinoid receptor antagonists SR141716A (rimonabant, trade name Accomplia) and SR144528, respectively [14, 15]. The initial importance of these antagonists rested with their usefulness as tools in determining receptor mediation of cannabinoid effects. Subsequently, it was reported that SR141716A acted as an inverse agonist in certain *in vitro* tests, particularly at higher concentrations [16]. *In vivo*, SR141716A blocks most of the cannabinoid effects of plant-derived and synthetic cannabinoids; however, it also produces locomotor stimulation (opposite of the locomotor suppression produced by active cannabinoids) [17]. Tests

with a series of structural analogs of SR141716A indicated that this stimulatory effect did not show a systematic structure–activity relationship; hence, it is probably not CB₁ receptor mediated [18]. Currently, SR141716A has been approved in the United States as an anti craving agent for smoking cessation and is undergoing phase III clinical trials as an appetite suppressant.

While the initial focus of marijuana research was on understanding the abuse potential of this drug, increasing interest has been shown in medical marijuana as well as in the therapeutic implications of pharmacological manipulations of the endocannabinoid system through which marijuana acts. The possible physiological roles of this system are only now starting to be delineated. In the following sections, we describe the endocannabinoid system and its potential physiological roles as well as discuss the ways in which marijuana may interact with this system.

18.2 ENDOCANNABINOID SYSTEM

The endocannabinoid system is one of several lipid signaling systems in the brain and in the body. This system consists of four basic elements: (a) two identified receptors, (b) several derivatives of arachidonic acid that serve as endogenous ligands, (c) synthetic and degradative pathways for these endocannabinoids, and (d) signal transduction pathways. Each of these components contributes to the overall functioning of the signaling system and is discussed in further detail below. In addition, the endocannabinoid system is not static over the course of development. For this reason, a brief description of age-dependent changes that occur during early life is included.

18.2.1 Cannabinoid Receptors and Signaling

To date two cannabinoid receptors, CB₁ and CB₂, have been identified. A splice variant of the CB₁ receptor, CB_{1A}, has also been cloned [19], but its biological significance remains unknown. CB₁ receptors are located primarily in the brain (as discussed in greater detail later). In contrast, CB₂ receptors are located primarily (but not exclusively; see [20]) in the periphery and are involved in the immunoregulatory effects of cannabinoids [21, 22]. Δ^9 -THC binds with approximately equal affinity to both subtypes of receptors [23]. Recent research results have raised the possibility that additional non-CB₁, non-CB₂ receptor(s) may exist, although none has been definitively identified (see [24] for a review).

The largest concentration of CB₁ receptors is located in the central nervous system, although their presence has also been noted in other parts of the body. Autoradiographic studies have shown high levels of CB₁ receptor binding in the basal ganglia (substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, and lateral caudate putamen) and the molecular layer of the cerebellum [25]. Cannabinoid-induced motor impairment may be related to activation of CB₁ receptors in these regions. Intermediate levels of binding are present in the CA pyramidal cell layers of the hippocampus, the dentate gyrus, and layers I and VI of the cortex. These receptors are most likely responsible for cannabinoid effects on memory and cognition. CB₁ receptors are also present in the ventromedial striatum and nucleus accumbens, areas that are associated with dopamine and mediation of brain reward. Localization here is consistent with the observation that most drugs of

abuse (including marijuana) directly or indirectly modulate dopamine release in these brain regions. Sparse levels were detected in the brain stem, which likely explains the low respiratory depressant effects of cannabinoids.

The predominant centrally mediated effects of cannabinoids occur through activation of inhibitory G proteins ($G_{i/o}$ and/or G_i) coupled to CB_1 receptors [26], although there was a report that CB_1 receptors can couple to G_s proteins [27]. Binding and activation of CB_1 receptors by Δ^9 -THC, anandamide, and other cannabinoid agonists results in a number of intracellular signaling processes, including inhibition of adenylyl cyclase, inhibition of calcium channels (N and Q types), and activation of inwardly rectifying potassium channels [28, 29]. In addition, mitogen-activated protein kinases are activated by the CB_1 receptor [30]. Whether all signal transduction systems are activated simultaneously is not known nor is the extent to which they are involved in specific cannabinoid actions. In addition, recent evidence has demonstrated the presence of an allosteric binding site on CB_1 receptors that modulates the affinity and efficacy of exogenous cannabinoids [31]. The effects of binding to this site on modulation of endocannabinoid activity under physiological conditions remain unknown.

In most traditional neurotransmitter systems (e.g., monoamines), receptors may be either pre- or postsynaptic; however, one-to-one correspondence between specific neurons and the neurotransmitter that they release is the general rule. A unique aspect of the endocannabinoid system is that endocannabinoids are released from neurons that are associated with release of many other neurotransmitters, including dopamine, γ -aminobutyric acid (GABA), and glutamate [32]. CB_1 receptors are prevalent on presynaptic terminals of neurons associated with almost all known neurotransmitters. Activation of these receptors is modulatory with respect to release of the primary neurotransmitter. The complexity of cannabinoid modulation of neurotransmitter release is further illustrated by recent findings that endogenous cannabinoids may act in a retrograde signaling fashion [33]. In this process, depolarization of a postsynaptic neuron elicits release of an endocannabinoid such as anandamide from that neuron. Endocannabinoids have a direct inhibitory action on release; however, their presynaptic localization on neurons that release both excitatory and inhibitory neurotransmitters means that the end result of CB_1 receptor activation may be either inhibitory [if release of an excitatory neurotransmitter such as glutamate is inhibited; i.e., depolarization-induced suppression of excitation (DSE)] or excitatory [if release of an inhibitory neurotransmitter such as GABA is inhibited; i.e., depolarization-induced suppression of inhibition (DSI)]. Indeed, inhibition of release of a neurotransmitter that tonically regulates the release of another neurotransmitter can result in increased release of the latter. For example, cannabinoids are also known to stimulate the release of dopamine in the nucleus accumbens by inhibition of glutamate release.

18.2.2 Synthesis and Metabolism of Endocannabinoids

To date, at least three arachidonic acid derivatives that serve as endocannabinoid ligands have been identified: arachidonylethanolamide (anandamide [9]), 2-AG [34], and 2-arachidonoyl-glyceryl ether (noladin ether [35]). Two other endocannabinoid ligands, *N*-arachidonoyldopamine [36] and *O*-arachidonylethanolamine (virodhamine) [37], have also been proposed. Anandamide, by far, is the best characterized.

In the brain, anandamide binds to and activates CB₁ receptors, which, as described above, are G-protein-coupled receptors. Anandamide also interacts with transient receptor potential V1 (TRPV1) receptors and with a number of cation channels [38–40].

Synthetic and degradative pathways for endocannabinoids have been identified. Substantial evidence indicates that anandamide is formed from arachidonic acid that is bound to cell membranes. This process is calcium and energy independent and involves activation of a transacylase that transfers arachidonic acid from the *sn*-1 position of phosphatidylcholine to the amino group in phosphatidylethanolamine, with subsequent hydrolysis by a phospholipase-D-type enzyme [41]. Since anandamide is not stored in vesicles, it is synthesized and released on an “as-needed” basis. Inactivation of anandamide occurs primarily via degradation by fatty acid amide hydrolase (FAAH), an enzyme that also degrades a number of other endogenous fatty acids, including the sleep-inducing lipid oleamide [42]. This enzyme has been cloned [43]. In mice, potentiation of the actions of exogenously administered anandamide is observed with pharmacological blockade of FAAH with a FAAH inhibitor and with deletion of the gene encoding for FAAH [44]. Anandamide may also be inactivated in part through a specific uptake mechanism in which it is transported across cellular membranes by a protein-mediated process that has the characteristics of facilitated diffusion [i.e., bidirectional and sodium and adenosine triphosphate (ATP) independence] [45].

18.2.3 Development

In humans, most of the research into the effects of marijuana on development has concentrated on identification of possible marijuana-induced fetal and birth defects. To this end, epidemiological studies have revealed that the most consistent effect of maternal marijuana use is shortened gestation and reduced birth weights; however, few long-term developmental consequences have been noted in the offspring of mothers who smoked marijuana during pregnancy [46]. By and large, the effects that have been reported are subtle and most appear to be reversible. The major exception appears to be higher cognitive functioning (executive functioning) that is impaired for a sustained period of time in the children who were exposed in utero.

Much of what we know about the acute and long-term effects of marijuana on the developing brain was discovered in the course of preclinical research with Δ^9 -THC in rodents. Prenatal exposure studies in rodents have shown that high doses of cannabinoids can produce resorption, growth retardation, and malformations, but only at doses that also produced malnutrition, which has similar consequence [47]. Doses of Δ^9 -THC that do not alter maternal body weight produce little effect on fetal development. The results of a number of studies of postnatal exposure to Δ^9 -THC in rodents have also been published. Since rats are born at a more immature stage than are humans, the preweanling period of a rat's life most closely corresponds to prenatal development in humans. During the first month or so of a rat's life, the endocannabinoid system in the brain undergoes a number of important changes. CB₁ receptors in the rat brain exhibit a progressive increase in number, but not in affinity, during the preweanling period (i.e., before postnatal day 21) and during early adolescence (females peak at postnatal day 30 and males peak at postnatal day 40). Receptors are pruned and decline to adult levels during later adolescence [48–50]. By postnatal day 60, adult levels of cannabinoid receptors are achieved [48]. Increases

in levels of the endogenous cannabinoid anandamide and *N*-arachidonoyl-phosphatidylethanolamine (an anandamide precursor) accompany these changes in receptor number throughout development [51]. While most brain CB₁ receptors present during development are distributed similarly to adult receptors (e.g., high levels in striatum, cerebellum, and cortex), an early transient atypical localization in pre-synaptic white matter has also been observed, suggesting a role for the endogenous cannabinoid system in neuronal migration and brain development [52, 53]. In addition, functional brain CB₁ receptors and anandamide also play a crucial role in physical growth and development, as they are necessary for milk suckling [54]. Although similar experiments obviously cannot be performed in human children and adolescents, it has recently been recognized that the human brain also undergoes substantial reorganization during adolescence [55].

18.3 MARIJUANA PHARMACOLOGY: IMPLICATIONS FOR PHYSIOLOGY OF ENDOCANNABINOID SYSTEM

Marijuana is typical of most psychoactive substances in that it produces a broad array of behavioral and pharmacological effects, many of which are subjective and differ among users. Some of the more prevalent effects on the central nervous system include euphoria, sedation, dreamlike state, distorted perceptions of sensory information and time, disrupted cognition, and impairment of fine motor skills [56]. Δ^9 -THC produces the subjective "high" associated with smoking marijuana and represents the chemical basis for many of its other effects in the central nervous system.

In rodents, Δ^9 -THC, other plant-derived cannabinoids, and synthetic cannabinoids that bind to CB₁ receptors with reasonable affinity produce a characteristic tetrad of pharmacological effects in mice, including suppression of locomotor activity, hypothermia, antinociception, and catalepsy [57]. These psychoactive cannabinoids also produce Δ^9 -THC-like discriminative stimulus effects in rats and rhesus monkeys (for a review, see [58]). Among this group of cannabinoids, potencies for producing these effects are highly correlated with affinities for the CB₁ receptor [5], suggesting that these effects are mediated via interaction with this receptor. Further evidence for CB₁ receptor mediation of these pharmacological properties is seen in blockade of the effects by SR141716A [17, 59], but not by administration of the CB₂ antagonist SR144528 [60].

Anandamide and structural analogs of anandamide produce a profile of pharmacological effects in mice that resemble those produced by Δ^9 -THC; however, anandamide-like cannabinoids have lower efficacies for effecting hypothermia: Body temperature decreases are about half that of traditional cannabinoids [61–63]. Further, correlations between in vitro affinities of anandamide analogs for CB₁ receptors and their in vivo potencies were not as strong as for other classes of cannabinoids [64, 65]. Differences in anandamide pharmacology also have been noted in drug discrimination studies [66] as well as in the mechanism through which anandamide produces spinal antinociception in mice [63, 67, 68]. In addition, it was reported that anandamide's pharmacological effects were not blocked by the CB₁ antagonist SR141716A, although SR141716A blocked the cannabimimetic effects of more stable anandamide analogs, such as 2-methyl-2'-fluoroethylanandamide [69].

An issue of concern with respect to these differences in anandamide's pharmacological effects is the extent to which pharmacokinetics may play a role. As mentioned previously, FAAH rapidly hydrolyzes anandamide to arachidonic acid [70, 71]. In contrast, Δ^9 -THC and other plant-derived cannabinoids are metabolized primarily through the hepatic P_{450} system [72], a process which requires much more time. For the most part, studies that have addressed the extent to which the observed differences in anandamide pharmacology might be related to these differences in its biodisposition have suggested that many of the anomalies in anandamide pharmacology (as compared to that of traditional cannabinoids) are not observed when the metabolism of anandamide is slowed, as in FAAH knockout mice [44] or as a result of administration of an agent that inhibits FAAH [73, 74]. Nevertheless, differences in anandamide pharmacology that cannot be explained by its rapid metabolism remain (see [13] for a review), suggesting that the effects of exogenously administered cannabinoids (including marijuana) may not entirely mimic physiological activation of the endocannabinoid system. This caveat should be kept in mind throughout the following descriptions of specific areas in which endocannabinoid involvement is strongly implicated and in which marijuana has prominent pharmacological effects.

18.3.1 Pain

Interest in cannabinoids as analgesic agents began with cannabis before attention turned to Δ^9 -THC and finally the endogenous cannabinoid system. Noyes et al. [75] demonstrated that orally administered Δ^9 -THC elevated mood, stimulated appetite, and produced some analgesia in cancer patients at doses that also produced dizziness, blurred vision, and impaired thinking. They concluded that Δ^9 -THC and codeine had comparable efficacy [76], while other investigators found it to have little analgesic efficacy [77, 78]. There has also been a conscientious effort to develop synthetic cannabinoid derivatives that might be useful as analgesics, but they too have been hampered by their behavioral side effects [79, 80].

Evaluations of cannabinoids in animal models have consistently shown them to be antinociceptive [81]. However, the fact that Δ^9 -THC analgesia is also accompanied by other effects, such as motor depression, raised questions regarding the validity of these antinociceptive measures. However, Walker et al. [82] showed that cannabinoids suppress nociceptive neurotransmission at the level of the spinal cord and the thalamus and that the effects were selective for painful as opposed to nonpainful somatic stimuli. Earlier, it had been shown that intrathecal administration of either the α_2 -noradrenergic antagonist yohimbine [83] or the κ -opioid antagonist nor-binaltorphimine (nor-BNI) [84] blocked cannabinoid-induced antinociception but failed to attenuate cannabinoid-induced motor impairment. Furthermore, intrastriatal administration of cannabinoids into the caudate nucleus produced catalepsy [85] without producing antinociception [86]. Cannabinoids have also been shown to exhibit antinociception in several chronic-pain models. The synthetic cannabinoid agonist WIN 55,212-2 produced anti-hyperalgesic responses following a chronic constriction injury of the sciatic nerve [87]. Another study that employed an arthritic pain model using Freund's adjuvant found Δ^9 -THC to be antinociceptive in both arthritic and nonarthritic rats [88].

With the identification of the endogenous cannabinoid system, it became possible to establish the mechanism of cannabinoid-induced analgesia and to further delineate

between direct and indirect effects. Initial studies showed that exogenous administration of anandamide to mice [63, 89] and rats [90] was antinociceptive. This evidence coupled with observations that cannabinoids inhibit nociception [86, 91] when injected into brain areas associated with antinociception [92] and that contain cannabinoid receptors [93] suggested the presence of a cannabinoid system that functioned to modulate pain. Electrical stimulation of periaqueductal gray (PAG) induced CB₁-mediated analgesia and simultaneously released anandamide [94]. Also the injection of formalin into the paw induced a nociceptive response and concomitant release of anandamide from the PAG. In fact, an earlier investigation had suggested that an endocannabinoid tone may downmodulate pain perception via CB₁ receptors in another region of the brain stem, the rostral ventromedial medulla, through the same circuit previously shown to contribute to the pain-suppressing effects of morphine [95]. Calignano et al. [96] suggested an endocannabinoid and CB₁/CB₂-mediated tone controlling pain at the peripheral level, mostly based on the observation that local administration of the antagonist for each receptor subtype led to hyperalgesia, whereas exogenous anandamide blocked the painful response of mice to formalin injection. In a subsequent study, however, no difference with vehicle-treated rat paws was found in the amounts of anandamide and 2-AG in the hind paw of rats after injection of formalin and during the maximal nociceptive response [97]. These studies, taken together with that by Meng et al. [95] and Walker et al. [94], suggest that if endocannabinoids tonically modulate inflammatory pain perception they may do so at sites different from those of inflammation.

It is also well known that stress will induce antinociception. Suplita et al. [98] demonstrated that a descending cannabinergic neural system is activated by stress to modulate pain sensitivity in a CB₁ receptor-dependent manner. Furthermore, this pathway involves both brain stem rostral ventromedial medullar and midbrain dorsolateral PAG. Stress produces an elevation in both anandamide and 2-AG levels in the PAG, and SR141716 blocks the associated stress-induced analgesia [99]. These latter investigators provided further evidence for endocannabinoid involvement by demonstrating that inhibitors of monoacylglycerol lipase (degrades 2-AG) and FAAH (degrades anandamide) also enhance stress-induced antinociception when injected into the PAG. It had been shown previously that FAAH inhibitors greatly elevated anandamide levels in rodent brain and produced CB₁ receptor-dependent antinociception [100]. As mentioned above, it is logical to presume that a CB₁ receptor antagonist might produce hyperalgesia if elevation of anandamide produces antinociception. However, one study reported that SR141716 produced hyperalgesia in the hot-plate test [101], whereas another failed to show hyperalgesia to a mechanical stimulus in either nonarthritic or arthritic rats [88].

The first electrophysiological evidence that cannabinoids can block spinal pain pathways was the intravenous administration of WIN 55,212-2 that selectively suppressed noxious-evoked firing of the wide-dynamic-range (WDR) neurons to a noxious pressure stimulus [102]. Importantly, WIN 55,212-2 did not affect the stimulus-evoked activity of non-nociceptive neurons in the spinal cord. WIN 55,212-2 also inhibited noxious stimulus-evoked activity of neurons in the ventrolateral posterior nucleus of the thalamus [103]. Although these results demonstrated that cannabinoids suppress the ascending nociceptive pathway, they did not address the site of cannabinoid action in the central nervous system (CNS). Experimental evidence indicates that cannabinoids inhibit nociceptive responses at both spinal and

supraspinal sites. Intravenous administration of either Δ^9 -THC or CP 55,940 to spinally transected rats was found to reduce, but not eliminate, antinociception [104]. Direct injections of cannabinoids into the brain provided evidence for a supraspinal site of action [86, 105]. Direct administration of WIN 55,212-2 into the dorsolateral PAG or dorsal raphe nucleus, but not the ventral PAG, medial septal area, lateral habenula, arcuate nucleus, or perihypothalamic area, produced a partial antinociceptive effect [91]. Similarly, CP 55,940 infused into the posterior ventrolateral area in the region of the dorsal raphe produced antinociception as well as other pharmacological actions [86]. In contrast, drug administration into either dorsolateral or anterior ventrolateral PAG sites or outside of the PAG borders was without effect.

Many antinociceptive drugs acting in the brain activate descending neurochemical systems to inhibit the input of noxious stimuli at the spinal level. Spinal noradrenergic and serotonergic fibers are believed to play a predominant role in the antinociceptive effects of a variety of drugs. There is evidence suggesting the involvement of monoaminergic systems in cannabinoid-induced antinociception. Both 5,7-dihydroxytryptamine [106], a serotonergic neurotoxin, and 6-hydroxydopamine [107], a dopaminergic neurotoxin, reduced the antinociceptive effects of cannabinoids. Other evidence implicating the involvement of norepinephrine in cannabinoid-induced antinociception is that intrathecal (i.t.) administration of the α_2 -noradrenergic antagonist yohimbine blocked the antinociceptive effects of intravenous (i.v.)-administered Δ^9 -THC [83]. In contrast, i.t. injection of the nonspecific serotonin antagonist methysergide failed to reduce Δ^9 -THC-induced antinociception. These findings are consistent with the hypothesis that the supraspinal component of cannabinoid-induced antinociception is mediated through a descending spinal noradrenergic system. There is also evidence that GABA_B receptor agonists produce antinociception through modulation of the endocannabinoid system at the spinal level. SR141716 blocked the antinociceptive effects of baclofen but the, GABA_B antagonist saclofen did not block the analgesic effects of CP55940 [108].

Recently, attention has turned to the CB₂ receptor as an important target for regulation inflammation and the pain associated with it. The CB₂ selective agonist HU-308 reduced formalin-induced pain and arachidonic acid-induced inflammation (ear swelling) [109]. Another CB₂ selective agonist, AM1241, has been reported to be anti-inflammatory and analgesic in several pain models [110]. The nonselective agonist CP 55940 was reported to be effective in a neuropathic pain (spinal nerve ligation) model, and its effects were blocked only by the CB₂ receptor antagonist [111]. Valenzano et al. reported that a selective CB₂ receptor agonist was active in neuropathic, incisional, and chronic inflammatory pain models and was inactive in CB₂ receptor knockout animals [112]. Moreover, CB₂ receptors were upregulated in dorsal root ganglia (DRG) following sciatic nerve section or spinal nerve ligation [113].

TRPV1 represents another possible target for cannabinoid action. Jerman et al. demonstrated that anandamide caused a concentration-dependent increase in intracellular calcium concentrations in VR1-HEK (human embryonic kidney) and DRG cells that was blocked by capsazepine but not by SR141716 [114]. The authors concluded that anandamide analgesic properties are likely to be mediated at least in part through TRPV1 activation. Lam et al. also found that anandamide was a partial agonist in increasing intracellular concentrations in TRPV1-containing HEK cells, but they did not report whether its effects were blocked by capsazepine [115].

Unfortunately, there is little *in vivo* evidence that anandamide antinociception is mediated through TRPV1, in part because TRPV1 antagonists are typically not very effective when administered to animals. Capsazepine does not attenuate cannabinoid stress-induced analgesia [98]. There are suggestions that hybrid anandamide/capsaicin analogs may produce some of their antinociceptive effects through TRPV1, but direct evidence is lacking [116].

18.3.2 Cognition

One of the most prominent behavioral effects of Δ^9 -THC and other psychoactive cannabinoids is disruption of cognition. This effect has been observed in humans and in animal models. In human marijuana users, smoked marijuana interferes with the ability to learn and recall verbal information and it impairs short-term memory [117–119]. In addition, smoked marijuana disrupts timing ability in experiments requiring subjects to estimate elapsed time. Subjects in these experiments responded prior to completion of the specified time interval, suggesting overestimation of elapsed time [120, 121]. Similar impairments of short-term (i.e., working) memory and timing ability have been observed in animal models (see [122] for a review). Rodents injected with an acute dose of a psychoactive cannabinoid exhibited impairments in recognition memory as well as delay-dependent deficits in accuracy in delayed match/nonmatch to sample procedures and in spatial water and land maze tasks [123–126]. CB₁ receptor mediation is indicated, as these effects were reversible upon elimination of the drug or administration of SR141716A and were not evident in CB₁ knockout mice [127]. Interestingly, memory impairment produced by Δ^9 -THC in a delayed match-to-sample task was accompanied by decreases in firing rates of hippocampal neurons during the sample, but not the match, phase of the experiment, suggesting that, at least for this task, Δ^9 -THC disrupts encoding of memories during the sample phase, but not their retrieval during the match phase [124]. Δ^9 -THC-induced dopamine hyperactivity in the prefrontal area also has been implicated in some types of observed memory impairments [128]. While cannabinoids affect acquisition (i.e., learning) to a lesser degree than they do working memory, acquisition of new information *is* impaired. For example, Δ^9 -THC increased the number of errors made by rats working in a repeated acquisition task [129]. In contrast, long-term memory is relatively unaffected in both humans [130] and animals [122] following cannabinoid administration, suggesting cannabinoid interference with only certain types of cognition.

In addition to inducing selective cognitive impairments, Δ^9 -THC is reported to produce severe disruption of timing and temporal discrimination in rodents. Consistent with results of human studies with smoked marijuana [120], peripherally injected Δ^9 -THC impaired performance of rats responding for food reward under differential reinforcement of low rates (DRL) operant schedules [131, 132]. In this type of schedule, an animal is required to wait for a specified length of time since the last response before responding will again be reinforced; that is, the interresponse time (IRT) must be of a specified minimum length. Under conditions in which the specified IRT was short (5 s or less), Δ^9 -THC had no effect on the pattern of responding; however, when the target wait time was lengthened to 10–15 s, premature responding was observed. Collectively, the results of both human and rodent studies suggest that Δ^9 -THC disrupts timing by making longer delays seem shorter.

Conversely, Han and Robinson [133] found that SR141716A increased estimated time in another type of operant procedure, raising the possibility that it may do so by blocking endogenous anandamide.

Cannabinoid effects on learning, working memory, and temporal discrimination occur within the range of doses that are intoxicating in humans and that produce discriminative stimulus effects in animals, but at doses lower than those required to elicit other characteristic cannabinoid effects (e.g., motor suppression, analgesia, and hypothermia). This separation of effects suggests a degree of selectivity for cannabinoid effects on higher functioning.

Results of studies that involved CB₁ receptor nullification (e.g., pharmacological blockade, genetic manipulation) have suggested a possible physiological role of endocannabinoids in cognition. Selective antagonism of CB₁ receptors with SR141716A delayed extinction in a previously learned water maze task in mice at doses that did not affect swim speeds, and mice lacking CB₁ receptors exhibited a similar delay in extinction in this task [127]. In each case, CB₁ receptor nullification increased perseveration in a previously learned behavior. These findings may result from interference with cognitive processes related to forgetting (e.g., memory duration) and/or to suppression of behavior (i.e., extinction). If increased memory duration were the primary factor effecting perseveration, the delay between acquisition of a behavior and its recall would be most important to performance (i.e., perseveration would occur at short delays and would be independent of number of trials). In contrast, a primary deficit in extinction would evince less perseveration with increasing number of trials. In a study that varied number and spacing of trials, SR141716A-induced perseveration was most apparent in extinction trials that occurred infrequently (spaced trials) versus those that occurred consecutively on the same day (massed trials) [134]. Although these results seem to suggest that SR141716A's primary effect was on extinction, its effects on forgetting could not be assessed adequately due to the extended duration of memory in the control group. Given that SR141716A produced its pharmacological effects on cognition at doses that have not been associated with inverse agonism at CB₁ receptors, antagonism of endocannabinoid action at these receptors is the most likely mechanism. However, while it is tempting to speculate that endocannabinoids tonically modulate the neural pathways that underlie cognition, empirical results supporting this hypothesis are still inconclusive.

18.3.3 Appetite Regulation

Marijuana increases appetite in humans, which was recognized even in historical accounts. Indeed, this effect serves as the basis for development of oral formulations of Δ^9 -THC that are currently in therapeutic use to treat cachexia in cancer and AIDS patients [135, 136]. As in humans, Δ^9 -THC and other psychoactive cannabinoids increase food intake in rodent models, as does exogenously administered anandamide [137–139]. This effect is blocked by coadministration of SR141716A (but not SR144528) and is not observed in CB₁ knockout mice [140–142]; hence, it appears to be CB₁ receptor mediated. Further, SR141716A and several of its analogs produce effects on feeding that are opposite those of cannabinoid agonists; that is, they dose-dependently decrease food consumption in rodents [141, 143, 144], and in clinical trials in humans SR141716A produces weight loss [145].

In humans and other mammals, food intake is a complex physiological process that is regulated by both homeostatic mechanisms and the hedonic value of food. Endocannabinoid involvement is implicated in both types of regulation [146], as anandamide levels in both the hypothalamus (associated with homeostatic mechanisms) and limbic forebrain (associated with hedonics of food intake) are increased in hungry rats and return to basal levels when rats are satiated [147]. The hypothalamus is a major player in homeostatic control of appetite, and it is here where endocannabinoids exert their primary central effects on this type of regulation of food intake. Although the hypothalamus does not have many CB₁ receptors as compared to other areas rich in these receptors (e.g., hippocampus), those that it has appear to be very efficient at activating intracellular messenger systems. An increase in hypothalamic anandamide levels, through either endogenous release or exogenous administration directly into the hypothalamus, acts to stimulate eating [148].

Of course, the endocannabinoid system is but one of the many neuromodulatory systems that affect food intake (e.g., [149]). For example, the hormone leptin is a key regulator of feeding behavior that is found mainly in white adipose tissue. Upon feeding, it is released into the circulation. Once leptin reaches the hypothalamus, it initiates a series of signaling events that eventually leads to release of anorexigenic peptides (e.g., pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript) or orexigenic peptides (e.g., neuropeptide Y and agouti-related protein). Leptin also exerts negative control over levels of anandamide and/or 2-AG in the hypothalamus [140]. Further, these investigators found higher levels of hypothalamic endocannabinoids in obese mice and rats with congenitally disrupted leptin signaling.

Endocannabinoids may also play a direct role in lipid metabolism in the periphery via activation of CB₁ receptors in adipocytes [150]. Blockade of these receptors by rimonabant stimulated Acp30 (adiponectin) messenger RNA (mRNA) expression in adipose tissue and reduced hyperinsulinemia in obese (*fa/fa*) rats [151]. These findings suggest that inactivation of the endocannabinoid system may aid weight loss by altering energy balance and lipid metabolism, an hypothesis that has recently received additional support at the genomic level in a diet-induced obesity model in rats [152].

In addition to their roles in homeostatic control of eating, however, endocannabinoids also may be involved in regulation of the hedonic value of food (via action in brain areas associated with reward; e.g., nucleus accumbens and limbic forebrain). Levels of anandamide and 2-AG in these reward-associated areas were increased during food restriction and returned to baseline when rats were satiated, much as they were in the hypothalamus under similar conditions [147, 153]. Yet, other feeding-induced changes in endocannabinoids in these reward areas were not associated with similar changes in the hypothalamus. Whereas significant decreases in CB₁ receptor density in the hippocampus, cortex, nucleus accumbens, and entopeduncular nucleus were observed in rats fed palatable food to induce obesity, changes in the density of hypothalamic CB₁ receptors were not noted [154]. Down-regulation of cannabinoid receptors in reward areas in obese rats were hypothesized to be the result of prolonged elevation of endocannabinoid levels in these areas. The fact that these region-specific differences in CB₁ receptor density were selectively induced in obese rats by a taste-enhanced diet, and not by a normal laboratory rodent diet, suggests a special sensitivity to the hedonic aspects of food in these reward-associated areas. These areas also appear to mediate the hedonic effects of drugs of abuse (including marijuana), although the degree to which the rewarding

properties of food and drugs involve similar mechanisms within these areas is still unclear. Nevertheless, it is intriguing that SR141716A, which was originally marketed as a pharmacological aid for smoking cessation, has also been shown to be effective in decreasing intake of palatable food.

18.3.4 Neurotoxicity

There has been considerable interest in the influence of cannabinoids on neuronal excitability and toxicity that may have relevance to several disease states. Δ^9 -THC has been shown to have both convulsant and anticonvulsant properties depending upon the model. Δ^9 -THC will enhance kindling elicited by either chemical or electrical stimuli [155], whereas it will decrease maximal electroshock-induced tonic-clonic convulsions [156]. The observation that cannabidiol, a structurally related cannabinoid lacking affinity for CB₁ receptors, is also anticonvulsant [157] raises the question of mechanism of action. There are several lines of evidence indicating that the endogenous cannabinoid system is capable of regulating neuronal excitability. The anticonvulsant effects of Δ^9 -THC in the maximal electroshock procedure is blocked by the CB₁ receptor antagonist SR141716, while the anticonvulsant effects of cannabidiol are not [158]. Subsequent studies demonstrated that anandamide and one of its stable congeners were also anticonvulsant and that a CB₁ receptor antagonist lowered electroshock seizure threshold [159]. Collectively, these observations suggest disruptions in endogenous cannabinoid tone can result in altered neuroexcitability.

The endocannabinoid system also influences the rat pilocarpine model of epilepsy. Cannabinoid receptor agonists completely blocked spontaneous epileptic seizures in this model, whereas the CB₁ receptor antagonist SR141716A increased both seizure duration and frequency [160]. Furthermore, levels of 2-AG were increased significantly within the hippocampal brain region during seizures, and Western blot and immunohistochemical analyses revealed that CB₁ receptor protein expression was significantly increased throughout the CA regions of the hippocampus of epileptic animals [160]. Similar results were obtained in animals lacking CB₁ receptors in that they exhibited increased kainic acid-induced seizures, and kainic acid increased hippocampal levels of anandamide in wild-type mice [161]. These investigators concluded that the endogenous cannabinoid system provides on-demand protection against acute excitotoxicity in the CNS. One possible explanation for cannabinoid protective effects is the presence of CB₁ receptors on excitatory glutamatergic neurons. Activation of these receptors decreases glutamate release and therefore decreases excitotoxicity [162].

As for a possible explanation for convulsant effects of cannabinoids, it has been suggested that exogenous cannabinoids, such as Δ^9 -THC, might also activate CB₁ receptors on inhibitory GABAergic neurons, leading to a decreased release of GABA and a concomitant increase in seizure susceptibility [162]. There is also evidence that anandamide itself can be proconvulsive. FAAH(−/−) mice exhibit increased sensitivity to kainic acid [163]. Administration of anandamide dramatically augments the severity of kainic acid-induced seizures in FAAH(−/−) mice but not in wild-type mice and enhanced neuronal damage in the CA1 and CA3 regions of the hippocampus. These findings do not support a general neuroprotective role for endocannabinoids in response to chemical-induced excitotoxicity. On the other hand, inhibition of FAAH

by organophosphates did not lead to any overt neurotoxicity or change in behavior despite elevating anandamide levels [164].

A number of *in vitro* studies support an anticonvulsant mechanism of action for cannabinoids. WIN 55,212-2 attenuated low-magnesium-induced burst firing in hippocampal culture [165], and both anandamide and 2-AG decreased stimulation-induced population spikes and low-magnesium-induced epileptiform discharges in rat hippocampal slice preparations [166]. The mechanism underlying this dampening of excitability is believed to involve inhibition of presynaptic glutamate release [165]. However, in fever-induced seizures, there is an increase in the number of CB₁ receptors associated with cholecystokinin-containing perisomatic inhibitory inputs, no effect on glutamate release, and enhanced retrograde inhibition of GABA release [167]. These results suggest that inhibition of GABA rather than glutamate release is important for febrile seizures. *In vitro*, there is evidence that endocannabinoids act in a retrograde manner to produce depolarization-induced suppression of inhibition by suppressing GABA release and thus disinhibiting pyramidal neuronal activity [168]. As such it would be expected that endocannabinoids would be excitotoxic.

As for general neurotoxicity, Chan et al. reported that Δ^9 -THC caused shrinkage of neuronal cell bodies and nuclei and genomic DNA strand breaks [169]. Δ^9 -THC also stimulated release of arachidonic acid leading to speculation that Δ^9 -THC's mechanism for neurotoxicity involves arachidonic acid metabolism to prostanoid synthesis and generation of free radicals by cyclooxygenase. Δ^9 -THC induces apoptosis in cultured cortical neurons through activation of both c-Jun N-terminal protein kinase isoforms JNK1 and JNK2 [170].

On the other hand, endocannabinoids inhibit A β toxicity, and this protective effect was prevented by the CB₁ receptor antagonist [171]. Anandamide's effects appear to be mediated through the mitogen-activated protein kinase pathway. The observation that cannabinoids retained their antioxidative properties in cultured cerebellar granule cells derived from either CB₁ receptor knockout mice or control wild-type littermates suggests that the CB₁ receptor is not involved in the cellular antioxidant neuroprotective effects of cannabinoids [172]. The specificity of cannabinoid neuroprotection is subject to further question when non-CB₁ receptor cannabinoids such as cannabidiol also produce neuroprotection. Cannabidiol protects against hippocampal and entorhinal cortical neurodegeneration in a dose-dependent manner [173]. Earlier studies had also found Δ^9 -THC and cannabidiol to be equally effective in preventing hydroperoxide-induced oxidation [174].

The role of the endogenous cannabinoid system in neuronal excitation and toxicity remains to be resolved. It appears that the endocannabinoids are capable of exerting both protection and causation of toxicity depending upon the neuronal insult. This possible dual action may arise from the ability of endocannabinoids to alter the release of both excitatory and inhibitory neurotransmitters.

18.3.5 Emesis

Several animal studies indicate a direct role for endocannabinoid modulation of emesis. Darmani et al. [175] showed that CB₁ receptor agonists reduced cisplatin-induced emesis in the least shrew while the antagonist rimonabant produced the opposite effects. Similar findings were reported with cannabinoid agonists that attenuated lithium-induced vomiting in the musk shrew [176, 177]. In addition, combinations of

inactive doses of Δ^9 -THC and ondansetron were effective in blocking vomiting in the musk shrew [177]. The musk shrew has also been used to study conditioned retching, an animal model of anticipatory nausea and vomiting. Δ^9 -THC completely suppressed conditioned retching in this model [178]. In addition, cannabinoid agonists suppressed lithium-induced conditioned rejection, a model of nausea in rats [179]. Opioids are known to be powerful emetogenic agents. Activation of the cannabinoid system was also effective in blocking opioid-induced vomiting in ferrets [180]. CB_1 receptors were strongly implicated in that rimonabant blocked the action of cannabinoid agonists in this model. Importantly, Darmani et al. [181] found prominent CB_1 receptor binding in the nucleus tractus solarius of the shrew. Van Sickle et al. demonstrated that cannabinoid agonists inhibited emesis and retching in ferrets whereas CB_1 receptor antagonists potentiated emetic responses [182]. Moreover, these investigators found both CB_1 receptors and FAAH in dorsal vagal complex consisting of the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus in the brain stem. In addition, the CB_1 receptor was also found in the myenteric plexus of the stomach and duodenum. The antiemetic effects of Δ^9 -THC in ferrets were reported to be mediated selectively via the CB_1 receptor [183]. Evidence in the shrew suggests that cannabinoids act both presynaptically and postsynaptically in both central and peripheral serotonergic neurons to block emesis [184] as well as at D_2/D_3 dopaminergic receptors [185].

The recent discovery of CB_2 receptors in the dorsal motor nucleus of the vagus led to speculation that they may be involved in emesis [20]. However, CB_2 selective agonists failed to inhibit emesis in ferrets. On the other hand, 2-AG reduced emesis in ferrets, an effect that was attenuated by administration of both CB_1 and CB_2 antagonists, leading the authors to conclude that if CB_2 receptors are indeed involved in emesis, they require the presence of CB_1 receptors. Finally, a metabolically stable analog of anandamide blocked vomiting, whereas another endocannabinoid, 2-AG, was emetogenic in shrew [181], suggesting possible species differences in the effects of endocannabinoids on emesis.

As for clinical evidence, anecdotal reports of patients smoking marijuana to control chemotherapy-induced nausea and vomiting provided the initial clues. These reports led to clinical studies with Δ^9 -THC in which it was found to be useful in patients whose chemotherapy-induced nausea and vomiting were refractory to other standard antiemetics available at that time [186]. Plasse et al. [187] reported that combinations of Δ^9 -THC and prochlorperazine resulted in enhancement of efficacy as measured by duration of episodes of nausea and vomiting and by severity of nausea. In addition, the incidence of psychotropic effects from Δ^9 -THC appeared to be decreased by concomitant administration of prochlorperazine. The combination was significantly more effective than was either single agent in controlling chemotherapy-induced nausea and vomiting [188]. Nabilone, a synthetic derivative of Δ^9 -THC, was also reported to be an effective oral antiemetic drug for moderately toxic chemotherapy [189]. Cannabinoids have also been found to be effective in treating nausea and vomiting in children undergoing chemotherapy [190, 191]. As for the current status of antiemetics, serotonergic antagonists such as ondansetron have become the standards for managing emesis. These agents have proven to be effective in preventing chemotherapy-induced nausea and vomiting in most patients. However, delayed nausea and vomiting are less well controlled. Therefore, the search for more effective agents continues. Combination therapy with ondansetron and

Δ^9 -THC has not been fully explored. In addition, there is a need for a higher efficacy CB₁ receptor agonist with fewer side effects.

18.4 ENDOCANNABINOID ROLE IN REWARD, TOLERANCE, AND DEPENDENCE

With the exception of medical marijuana use, most people who have experienced the psychoactive effects of cannabinoids did so through voluntary self-administration of smoked or oral marijuana for the sole purpose of becoming intoxicated. Laboratory animals (including rats, mice, pigeons, and monkeys) can also detect the distinctive psychoactive effects of cannabinoids in drug discrimination, an animal model of these subjective effects [6, 58]. Moreover, monkeys and rats find cannabinoids reinforcing and will self-administer them under appropriate experimental conditions [192–194]. SR141716A blockade of the discriminative stimulus and reinforcing properties of cannabinoids in both humans and animals implicates direct CB₁ receptor mediation in cannabinoid intoxication and reward [194–196]; however, as with most other abused substances, indirect alteration of dopamine neurotransmission in brain reward areas [e.g., ventral tegmental area (VTA) and nucleus accumbens (NAc)] is also involved (for a review, see [197]).

Several mechanisms through which neural functioning in reward-related areas is affected by cannabinoids have been identified. First, peripherally administered Δ^9 -THC directly increased dopamine levels in the NAc in a calcium-dependent manner, an effect that was blocked by SR141716A as well as by preventing generation of action potential by administration of the sodium channel blocker tetrodotoxin [198, 199]. Second, Δ^9 -THC preferentially increased burst activity of dopamine neurons in the VTA [200, 201]. Since CB₁ receptors are not localized in the VTA, this increase is probably mediated via disinhibition of local GABA circuitry [202, 203], perhaps by a mechanism similar to that responsible for depolarization-induced suppression of inhibition. Third, although the exact mechanism is unclear, opioid mechanisms also play a role in cannabinoid modulation of reward pathways, as indicated by reversal of cannabinoid effects by administration of the opioid antagonists naloxone and naloxonazine [198, 204]. Interestingly, regulation of dopamine activity in reward-associated brain areas by cannabinoids may be bidirectional. Whereas research has shown that exogenous cannabinoids increase dopamine activity via several mechanisms (as described above), there is also evidence that dopamine activity affects endocannabinoid levels (anandamide and/or 2-AG) in limbic forebrain and prefrontal areas through intermediate glutamatergic mechanisms [205]. Further research is needed to elucidate completely all of the various mechanisms through which endocannabinoids play a role in the regulation of neural transmission in reward-related areas.

Although initial exposure to marijuana may be unpleasant, individuals who continue to use typically find the acute effects of marijuana rewarding, at least in the short term (most likely due to its effects on dopamine neurotransmission; see above). By definition, however, abuse involves repeated or chronic administration. Repeated administration of Δ^9 -THC and other psychoactive cannabinoids results in the development of profound tolerance (up to 100-fold) and cross tolerance to most cannabinoid effects in a number of animal species, including pigeons, rodents, dogs, monkeys, and rabbits (see [206] for a review). Pharmacodynamic events appear to

play the primary role in cannabinoid tolerance, as pharmacokinetic parameters (absorption, distribution, metabolism, and excretion) are relatively unaltered by chronic administration. Further, cross tolerance between Δ^9 -THC and anandamide-like cannabinoids has been reported to develop under certain circumstances, even though the primary metabolic pathways for these two classes of cannabinoids differ dramatically [207–209]. Pharmacodynamic tolerance is also indicated by the fact that the brains of cannabinoid-tolerant animals show profound CB₁ receptor down-regulation and reduced second-messenger signaling [210, 211].

Dependence also occurs with chronic exposure to cannabinoids, albeit it is somewhat more difficult to measure than for some other drugs of abuse (e.g., opioids). Dependence implies the presence of symptoms of withdrawal with abrupt termination following a period of chronic administration. Although a few reports have noted behavioral changes indicative of spontaneous withdrawal upon abrupt cessation of cannabinoids, heroic doses or continuous-infusion methods are typically required [212]. Most studies assessing cannabinoid dependence have used precipitated withdrawal. In this approach, dependent animals that have been chronically treated with a psychoactive cannabinoid are administered an antagonist such as SR141716A. Symptoms of precipitated withdrawal in rats and mice chronically infused with Δ^9 -THC and then administered SR141716A included head shakes, facial tremors, tongue rolling, biting, wet-dog shakes, eyelid ptosis, facial rubbing, paw treading, retropulsion, immobility, ear twitch, chewing, licking, stretching, and arched back [213–215]. This syndrome was reversed by readministration of Δ^9 -THC [216]. These studies provide convincing evidence that cannabinoids can produce dependence and are consistent with clinical observations of marijuana dependence [217]. Nevertheless, the marijuana dependence syndrome is milder than that typically reported for opioids and psychomotor stimulants.

18.5 FUTURE DIRECTIONS

The discovery of the endogenous cannabinoid system makes it possible to conduct a systematic evaluation of the effects of marijuana and its constituents in order to discern their direct and indirect effects. It appears that most of the recreational and medicinal effects of marijuana are produced through the endogenous cannabinoid system. However, the discovery of this system has provided an exciting new avenue for exploring a wide range of physiological functions and new development strategies for treating disease. The endocannabinoid system is best described as a modulator of other systems, thereby adding to the complexity of defining its physiological roles. In the future, it is likely that our knowledge of the endocannabinoid system will expand with the identification of additional receptor subtypes and endogenous ligands and with a better understanding of how it modulates other biological systems.

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19

OPIATES AND ADDICTION

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19.1	Incidence and Prevalence of Heroin and Prescription Opiate Abuse	692
19.2	Treatment Statistics	694
19.3	Current Treatment Needs	696
19.4	Buprenorphine Studies	696
19.4.1	Buprenorphine and Buprenorphine/Naloxone: Studies for Food and Drug Administration Approval	696
19.4.2	Buprenorphine: Comparative Efficacy Studies	697
19.4.3	Buprenorphine: Implementation Issues and Pilot Studies	698
19.5	Future Challenges for Opiate Dependence Treatment	698
19.6	Drug Discovery Efforts for Opiate Dependence Treatments	699
19.7	Summary and Conclusions	700
	References	700

The abuse liability of opiates was recognized and written about as early as the sixteenth century. Abuse of and tolerance to opiates were described in manuscripts from Turkey, Egypt, Germany, and England [1]. Abuse of and dependence on opiates continue to represent a worldwide problem that is associated with significant morbidity and mortality. According to the World Health Organization, almost 13 million people abused opiate drugs in the years 1999–2001 [2].

Opiate abuse and dependence are associated with significant morbidity and mortality. Morbidity associated with opiate addiction includes the risk of contracting acquired immunodeficiency syndrome (AIDS) and hepatitis C infections from unsafe injection practices and high-risk sexual practices. The worldwide human immunodeficiency virus (HIV) epidemic continues at a rapid pace. Of the estimated 40 million people infected with HIV, 5 million were infected during 2003. In regions of the world where the infection is spreading the fastest, drug injectors and their sexual partners account for most of the new infections [3]. The U.S. Centers for Disease Control and Prevention (CDC) estimates that 36% of the AIDS cases in the United States are due to injection drug use or sexual activity with an injection drug user. Chitwood et al. [4] compared HIV infection prevalence across new injectors, long-term injectors, and heroin sniffers. The new injectors and the heroin sniffers had

prevalence rates of 13.3 and 12.7%, respectively, whereas the long-term injectors had a 24.7% prevalence rate. The attributable HIV risk from injection was 5.8% for the new injectors versus 55% for the long-term injectors. These data suggest that sexual risk factors play a major role in the spread of HIV into the general population in the new-injector and sniffer groups. The injection drug-using population is a significant contributor to the AIDS epidemic, suggesting that curtailment of the epidemic will not occur until this population is successfully treated for drug use and effectively counseled regarding unsafe sexual practices. Moreover, noninjection heroin users who are HIV positive also can contribute to the HIV epidemic by sexual transmission to their partners [5, 6].

Hepatitis C infections generally have a higher prevalence than HIV in injection drug users. Many infections occur early in the course of injection drug use. Garfein et al. [7] reported a 65% incidence of hepatitis C infection in a population with a one-year or less history of injection drug use. Over 95% of injection heroin users found to have significant liver disease in an early cohort (late 1970s and early 1980s) were later retrospectively found to have hepatitis C infection [8, 9]. Injection drug users infected with hepatitis C in this era will soon be or are already presenting with need for treatment and possible hepatic transplantation since 20% of hepatitis C-infected persons will progress to severe liver disease over a 20-year period of time. Of considerable concern, a very recent study has shown that essentially all of the former heroin addicts in methadone maintenance treatment who are HIV positive, and thus require treatment of AIDS infection, also have markers indicating prior or current hepatitis C infection [10]. Sixty-seven percent of the cohort were hepatitis C positive and 29% were HIV positive. Coinfection rates for the overall study group were 26%. HIV-hepatitis C virus (HCV) coinfection increases the progression of HCV-induced liver disease, resulting in faster progression to cirrhosis and end-stage liver disease [11]. Mortality increases from liver disease in HIV-HCV coinfecting patients have been reported [12].

“All-causes mortality” is high in untreated opiate-addicted populations. Desmond and Maddux [13] reported death rates in the illicit opiate-using population not in treatment ranged from 1.65 to 8.3% per year; the median yearly death rate was 3.5%. HIV disease has also impacted mortality and causes of mortality in this population. Quaglio et al. [14], analyzed heroin-related deaths in northeast Italy occurring from 1985 to 1998. Of the 2708 deaths, 37% were due to overdose and 32.5% were AIDS related. The mortality rate among the injection drug users (IDUs) was 13 times that of the general population. The effect of HIV infection on mortality in heroin users was also evaluated in the Vancouver Injection Drug Users Study [15]. Irrespective of HIV status, the leading cause of death was opiate overdose, accounting for 42% of the HIV-negative deaths and 25% of the HIV-positive cohort deaths. Of the 65 deaths among the HIV-positive group, 34% were AIDS related.

19.1 INCIDENCE AND PREVALENCE OF HEROIN AND PRESCRIPTION OPIATE ABUSE

The prevalence of heroin use is greater in developed countries; 2% of youth in western Europe, Canada, and the United States have tried heroin [2]. Moreover, the “purity” of heroin in the United States has increased significantly and the price has

fallen in the last 25 years from 7% in 1980 to an average of 51% in 2001 [16]. Heroin purity as high as 72% has been noted in Philadelphia. The change in purity has allowed a shift from intravenous use to intranasal use. The intranasal route is an acceptable route of administration for many heroin initiates. In fact, it is considered the method of choice for American adolescents [17]. Peak blood levels from intranasal use occur in 5 min, essentially equal to that seen with intramuscular injection [18]. The average age at which heroin was first used has dropped from 27 in 1988 to 19 in 1995 [17]. Use by the intranasal route tripled between 1991 and 1995. There is evidence that adolescent admission for heroin abuse and dependence tripled from 1993 to 1998 (see Section 19.2). One report noted that the average age was 17 years old for admission to treatment for heroin abuse [19]. For an overview of adolescent heroin abuse, see the review of Hopfer et al. [20]. There is also evidence that a significant percentage of heroin “snorters” will shift to injection [5, 6, 21].

Global statistics on the abuse of prescription opiates are unavailable. In the United States, incidence and prevalence rates of abuse of prescription opiates were obtained in the 2003 National Survey on Drug Use and Health (NSDUH) [22]. This survey is the primary source of information on illicit drug and alcohol use for the civilian, noninstitutionalized U.S. population aged 12 or older. In 2002, there were 2.5 million new users who endorsed nonmedical use of opiate analgesics. This is approximately a fivefold increase since 1990. (Another database, The Monitoring the Future study, a survey that questions 8th, 10th, and 12th graders about their drug use, has also noted a threefold increase in prescription opiate abuse since 1991 [23]). Fifty-five percent of the new users were females and 56% were age 18 or older. Individuals that were current users of opiate analgesics taken nonmedically totaled 4.7 million. It is doubtful that all of these individuals would meet the criteria of the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) for an opiate abuse diagnosis. In terms of lifetime prevalence, over 31 million Americans have used opiate analgesics in a nonmedical fashion. Opiate medications used most often in the survey were hydrocodone combinations (15.7 million), oxycodone combinations (10.8 million), hydrocodone (5.7 million), OxyContin (2.8 million), methadone (1.2 million), and Tramadol (186,000). When survey respondents were diagnosed using DSM-IV criteria, 57.4% of the heroin users met criteria for opiate abuse or dependence whereas 12.2% of the prescription opiate users met criteria for opiate abuse or dependence.

These data suggest that although there is a greater prevalence of prescription opiate abuse, the risk of dependence is lower in the population that abuses prescription opiate medications. Moreover, the data suggest an increasing problem with dependence on prescription opiates. Assuming one-eighth of the current nonmedical prescription opiate users meet DSM-IV criteria for abuse or dependence, that would equate to 600,000 people currently in need of treatment. Given the increasing incidence of nonmedical use of prescription opiates, the current situation is likely to worsen before it gets better.

Opiate abusers can be categorized into three groups: those abusing only heroin, those abusing only prescription opiates, and those abusing heroin and prescription opiates. An analysis of heroin, oxycodone, and heroin and oxycodone users was conducted in the 2003 NSDUH. Heroin and heroin-plus-oxycodone users had a 2:1 male-female ratio whereas the oxycodone-only group had more females (57:43). The majority of users of oxycodone, either alone or in combination with heroin, were

white (91%). Groups using heroin, either alone or in combination, were older than 35, whereas the majority (56%) of the oxycodone users were younger than 35. Past-year dependence diagnoses were 4, 16, and 7% for heroin, heroin plus oxycodone, and oxycodone alone, respectively.

In 2003, the NSDUH estimated that 19.5 million Americans were current illicit drug users (use within 30 days of the NSDUH survey). Of these, 3.8 million were considered to meet criteria for abuse or dependence and another 3.1 million were diagnosed with abuse or dependence to alcohol and illicit drugs. When alcohol use alone is added to these categories, 21.6 million people aged 12 or older were classified with a substance abuse disorder. Of this group, 3.3 million sought treatment in 2003. In terms of opiate users, 415,000 prescription opiate users and 281,000 heroin users sought treatment.

The treatment of opiate addiction can be conceptualized as occurring in three categories: discovery of new therapies, development of new therapies, and implementation of new therapies. The extant treatment system will be considered first so that the context of discovery, development, and implementation of new pharmacotherapies can be better understood.

19.2 TREATMENT STATISTICS

The Treatment Episode Data Set (TEDS) is another SAMHSA [24] data set that summarizes patient demographics and treatment characteristics of the 1.9 million patients admitted to treatment centers that report to individual state administrative systems. This system is not comprehensive but captures data that can be analyzed for year-to-year changes as well as long-term treatment trends.

As can be seen in Table 19.1, heroin admissions in the TEDS system increased from 1992 to 2002. In fact, heroin admissions accounted for 15% of the treatment admissions, making it the largest illicit drug for which individuals seek treatment. Admissions for adolescent heroin use tripled in the 1990s (TEDS 2000 data not shown). Male heroin users outnumber female users. A plurality of heroin users are white. Subtle shifts in racial composition of patients entering treatment and route of administration differences were also seen. The number of white heroin admissions increased from 1992, whereas the number of African-Americans had a slight drop (data not shown). Moreover, the data show a shift toward less injection use and more inhalation use among African-Americans whereas inhalation and injection use both increased among white heroin users. Additionally, the use of methadone as a treatment in this system appears to be declining for both injectors and inhalers.

In contrast, the overwhelming majority of prescription opiate users admitted into treatment are white. They also abuse the drug by the oral route of administration. The male-to-female ratio appears to be close to 1, suggesting a greater likelihood for females to be prescription opiate abusers. Although the number of treatment admissions represents only a small percentage of the treatment admissions in TEDS, the percentage has increased from 1992 to 2002. An even smaller percentage of these patients received methadone in their treatment.

Methadone was developed by in the 1960s as a treatment for heroin addiction (for a review, see [25]). There is documented evidence that methadone reduces morbidity

TABLE 19.1 Comparison of Treatment Facilities Admissions for Heroin Users and Prescription Opiate Users in 1992 and 2002 from TEDS

Patient Demographics and Use Variables	Heroin Admissions		Prescription Opiate Dependence Admissions	
	1992	2002	1992	2002
Number of admissions	170,370	285,657	13,671	45,605
Male, %	—	69	—	54
White				
Percent	—	48	—	88
Male/female ratio	—	31/17	—	47/41
Hispanic				
Percent	—	25	—	3.5
Male/female ratio ^a	—	17/4	—	1.5/1.0
African-American				
Percent	—	25	—	5
Male/female ratio	—	15.6/8.6	—	2.8/2.6
Median age	—	36	—	35
Injectors, %	77	62	25	13
Inhalation, %	20	33	3	9
Opiate smokers, %	1.4	3		3
Oral ingestion, %	0.7	2	66	75
Daily Opiate Use, %	—	80	—	68
Admissions to TEDS, %	10.9	15.2	0.9	2.4
Methadone therapy for injectors, %	61	38		
Methadone therapy for inhalers, %	44	32		
Methadone therapy, %	—	—	—	19

^aMexican and Puerto Rican admissions (excludes Cubans and other Hispanic groups).

and mortality associated with heroin addiction. The death rate falls dramatically for former heroin addicts in methadone maintenance treatment [26–28]. Methadone is primarily dispensed in specialty outpatient clinics [29]. The National Survey of Substance Abuse Treatment Services reported that 1215 of 13,428 facilities dispensed methadone or levomethadyl acetate (LAAM). On the survey reference date of October 1, 2000, 17% of the patients in the survey were reported to be in methadone treatment. There are a number of barriers to obtaining methadone. Patients entering treatment for detoxification from heroin actually receive methadone less often than patients receiving longer treatment (22 vs. 35%) [29]. For prescription opiate-dependent patients entered into the 2000 TEDS data set, those who worked full or parttime were more likely to receive methadone. No sex differences in receipt of methadone were seen in the TEDS system for prescription opiate users. Other potential barriers are lack of treatment facilities in rural areas. For example, admission rates for prescription opiate user treatment increased 155% in the United

States. The greatest increase (269%) was seen in nonmetropolitan areas (without a city population of 10,000 or more).

19.3 CURRENT TREATMENT NEEDS

To address the unmet treatment needs of heroin- and prescription opiate-dependent patients, several changes are needed. The first is a greater test of the concept of medical maintenance with methadone. Initial studies have shown that medical maintenance is not only feasible but can be highly successful with stable patients [30–33]. Expanded research with methadone in this model is necessary to continue the medicalization of the treatment of opiate dependence. Increasing access to the medical care system should have benefits to patients accessing medical care [34].

The second change needed is a greater uptake of opiate pharmacotherapy with buprenorphine (SUBUTEX) and buprenorphine/naloxone (SUBOXONE).

19.4 BUPRENORPHINE STUDIES

19.4.1 Buprenorphine and Buprenorphine/Naloxone: Studies for Food and Drug Administration Approval

Buprenorphine was recognized to possess pharmacological properties that could be utilized for treatment of opiate dependence [35]. Specifically, buprenorphine had an ability to block the effects of administered morphine while it had intrinsic opiatelike effects that would likely ensure better compliance than a nonopiate or narcotic antagonist. Its duration of action implied that it could be administered as a single daily dose. Buprenorphine was classified as a partial agonist of the μ receptor [36]. Its partial agonist properties produce a ceiling effect on respiration [37] that suggests a lower risk of severe respiratory depression or apnea. Moreover, the withdrawal syndrome reported was minimal [35].

Buprenorphine's efficacy was established in three large, prospective studies that formed the basis of its marketing approval. Johnson et al. [38] reported the initial efficacy of buprenorphine in the outpatient setting. In this study, the 8-mg sublingual buprenorphine liquid-per-day group had better retention and lower opiate use than an active control group receiving 20 mg of oral methadone. A second study reported that an 8-mg dose of sublingual buprenorphine liquid produced better retention and less opiate use than a 1-mg buprenorphine active-control group [39].

During the development of buprenorphine, Reckitt-Benckiser decided to market a solid dosage form of different tablet strengths. Moreover, it also agreed with National Institute on Drug Abuse (NIDA) that a second dosage form that contained naloxone would serve as an abuse deterrent. The two decisions to develop solid dosage forms of different tablet strengths and to add naloxone as an abuse deterrent necessitated additional research in the following areas: dosage form development, determination of the ratio of buprenorphine to naloxone, efficacy and safety of the tablet formulations, and bioequivalence of buprenorphine tablets to sublingual liquid. Dosage strengths of 2 and 8 mg were chosen. Several clinical pharmacology

studies were conducted in various opiate-dependent populations maintained on morphine [40, 41], methadone [42], or buprenorphine [43] to determine the ratio of buprenorphine to naloxone for the combination tablets. A 4:1 buprenorphine–naloxone ratio was chosen. A randomized, double-blind, placebo-controlled multicenter trial compared the buprenorphine tablet, the buprenorphine–naloxone tablet, and placebo in opiate-dependent patients [44]. Both tablets reduced opiate use in the first month of the study compared to placebo.

The U.S. Food and Drug Administration (FDA) approved buprenorphine (SUBUTEX) and buprenorphine/naloxone (SUBOXONE) in October 2002. These medications are indicated for the management of opiate dependence. As there are other medications used in the management of opiate dependence, the comparative efficacy of buprenorphine will be reviewed before addressing the implementation issues.

19.4.2 Buprenorphine: Comparative Efficacy Studies

The Cochrane Review Group recently reviewed the efficacy of buprenorphine versus placebo or methadone [45]. Thirteen studies were reviewed. Buprenorphine was judged to be superior to placebo in terms of retention at all buprenorphine doses, but high and very high doses were needed to show greater suppression of heroin abuse than placebo. In comparison to methadone, buprenorphine does not retain patients as well as methadone and does not suppress heroin use as well as methadone. Study design issues may confound the conclusion of inferior suppression of heroin use of buprenorphine. For example, there are two randomized, active controlled studies that allow flexible dosing with buprenorphine and methadone. Mattick et al. [46] randomized 405 opiate-dependent patients in three methadone clinics to buprenorphine or methadone in a flexible dosing design. There were no differences in illicit opiate use across groups, although retention in the buprenorphine group (50%) was less than that seen in the methadone group (59%, *n.s.*). Johnson et al. [47] randomized 220 patients to one of four medications groups: buprenorphine (16–32 mg), methadone low dose (20 mg), methadone high dose (60–100 mg), or levomethadyl acetate (75–115 mg). There were no differences in illicit opiate use in the buprenorphine versus methadone groups. The buprenorphine-treated group had a nonstatistically significantly lower retention (58%) than the high-dose methadone group (73%).

Gowing et al. [48] have recently reviewed the efficacy of buprenorphine for the management of opiate withdrawal. Buprenorphine was compared to methadone (three studies), clonidine (seven studies), or differing rates of dose tapering of buprenorphine. Buprenorphine-treated patients completed treatment more often than clonidine patients and with fewer adverse effects. No differences in completion rate were seen between buprenorphine and methadone, although buprenorphine may provide better symptom management. Gradual dose reduction of buprenorphine may assist patients in completing withdrawal. A recent study evaluated two dose regimens of buprenorphine tablets (2–4–8–4–2 or 8–8–8–4–2 mg/day) versus clonidine in the management of heroin withdrawal [49]. Both dose regimens suppressed withdrawal better than clonidine, but the high-dose buprenorphine group was superior to clonidine on such measures as drug craving.

19.4.3 Buprenorphine: Implementation Issues and Pilot Studies

While the final stages of development of the buprenorphine products were ongoing, the U.S. Congress passed the Drug Abuse Treatment Act (DATA) of 2000. This law allows qualified physicians to prescribe FDA-approved opiate products in Schedules III–V of the Controlled Substances Act (CSA) that are indicated for the treatment of opiate dependence. Both products were scheduled into C. III of the CSA. Qualified physicians can prescribe these products (as defined in the DATA) to 30 opiate-dependent patients in their medical practices. Physicians can be qualified by experience or training. Then they request a waiver to prescribe buprenorphine. To date, over 7000 physicians have received waivers.

Physicians in office-based practice can now prescribe buprenorphine. A consensus statement on office-based treatment of opiate dependence using buprenorphine has now been developed [50]. O'Connor et al. [51] reported that patients randomized to buprenorphine in a primary-care setting had greater retention and less opiate use than those randomized to a methadone clinic. Fiellin et al. [52] treated heroin-dependent patients with thrice-weekly buprenorphine in a primary-care center. Eleven of the 13 patients were retained through the 13-week study period. Nine of the 13 patients achieved at least 3 consecutive weeks of opiate-free urines. Gibson et al. [53] reported that retention and reduction of opiate use were equivalent in patients assigned to buprenorphine in a specialty clinic versus a primary-care setting.

Additional research on buprenorphine products has been conducted. The NIDA's Clinical Trials Network has conducted medically assisted withdrawal studies in clinic settings [54]. In this report, 234 buprenorphine/naloxone-treated patients were followed for 13 days; 68% completed the detoxification. Only 1 of 18 serious adverse events was considered possibly related to buprenorphine. The authors concluded that the use of buprenorphine/naloxone in diverse community settings was practical and safe. Lintzeris et al. [55] reported on an Australian study of the implementation of buprenorphine in community treatment settings by general practitioners and pharmacists. Patients were randomized to either buprenorphine or methadone in this study. Seventy-four percent of the methadone transfer and 46% of the heroin patients treated with buprenorphine were retained for six months in treatment. The authors concluded that buprenorphine could be safely delivered in the community setting.

19.5 FUTURE CHALLENGES FOR OPIATE DEPENDENCE TREATMENT

The NIDA will concentrate on the development of non-opiate-based treatments for opiate dependence. Naltrexone is approved for the prevention of relapse in formerly opiate-dependent patients. Although naltrexone has been shown to be superior to placebo for prevention of relapse in a criminal justice population [56], the major problem with naltrexone is adherence to therapy. One of the naltrexone depot dosage forms has been tested in an outpatient trial [57]. A clinical trial of a depot form of naltrexone in outpatients being treated for alcoholism was recently published [58]. It is anticipated that the development of this dosage form will continue. If and when the depot form of naltrexone is marketed for alcoholism, studies will commence in detoxified formerly opiate-dependent populations. Since adherence is the major problem with the use of naltrexone, it is anticipated that this dosage form would be an advance in treatment.

The management of opiate withdrawal by nonopioid medications is another medications development challenge. For example, lofexidine is a congener of the α_2 agonist clonidine. It has been tested in opiate-dependent populations undergoing withdrawal. The initial double-blind, placebo-controlled multicenter trial of lofexidine was halted due to overwhelming efficacy. Although buprenorphine has been shown to be superior to clonidine, the potential of a medication like lofexidine would be for managing withdrawal in settings where buprenorphine and methadone were not available.

Buspirone has been shown in an initial study to reduce opiate withdrawal symptomatology in patients undergoing detoxification from heroin or medically assisted withdrawal from methadone [59].

19.6 DRUG DISCOVERY EFFORTS FOR OPIATE DEPENDENCE TREATMENTS

The potential success in developing new treatments for opiate dependence will depend on translation of neuroscience-based treatments into new medications. Two of the most promising targets will be highlighted here. One of the most promising therapeutic targets is a corticotropin releasing factor (CRF) receptor antagonist. CRF has been shown to be involved in the mediation of responses involving arousal, affect, and aversion to negative situations (for a review, see [60]). CRF is involved in coordinating responses to internal or external threats to an organism's homeostasis. Drug withdrawal syndromes can be considered an aversive state. Writhing, chewing movements, "wet dog" shakes, salivation, lacrimation, diarrhea, and increased emotionality characterize opiate withdrawal in rats. The CRF-1 antagonist CP-154,526 has been shown to attenuate several signs of naltrexone-precipitated withdrawal in opiate-dependent rats [61]. The CRF-1 antagonist CRA 1000 attenuated naloxone-precipitated withdrawal signs in chronically morphine-treated mice [62]. The CRF-1 antagonist antalarmin reversed naloxone-induced place aversion in morphine-pelleted rats [63]. Lu et al. [64, 65] have reported a role for CRF in morphine relapse in animal models. These investigators reported that CP-154,526 blocked the reinstatement of footshock-induced morphine place preference. Shaham et al. [66] reported that CRF reinstated heroin self-administration and α -helical CRF blocked footshock-induced reinstatement of heroin self-administration. CP-154,526 also attenuated footshock-induced reinstatement of heroin self-administration in rats [67]. The aggregate results suggest that a CRF antagonist would attenuate opiate withdrawal and block conditioned aversion responses and stress-related lapses or relapses to opiates.

The cannabinoid antagonist SR141716A (rimonabant) has also shown promise as a potential medication for the treatment of opiate dependence in preclinical models. Rimonabant has been shown to decrease components of the opiate withdrawal syndrome in rats [68]. Rimonabant blocked morphine-induced place preference in mice [69]. In rats trained to self-administer heroin, rimonabant reduced self-administration in fixed-ratio [69] and fixed- and progressive-ratio conditions [70, 71]. Further, Devries et al. [71] showed rimonabant blockade of heroin-seeking behavior that was provoked by either a priming injection of heroin or heroin-associated cues. These data provide a strong rationale for testing of rimonabant in opiate-dependent patients. For further details, the reader is referred to LeFoll and Goldberg [72].

19.7 SUMMARY AND CONCLUSIONS

Abuse and dependence on opiate drugs are chronic problems. There have been some fundamental changes in abuse patterns in the United States, notably the increase in both heroin use and prescription opiate abuse in the last 10 years. The increase in heroin use has been fueled by the availability of concentrated heroin that can be used for inhalation or smoking. Adolescent heroin use has risen concomitantly with the higher concentration of heroin. The age of first heroin use has dropped by nine years, suggesting that a greater number of young people will become heroin dependent. Treatment for heroin abuse is currently the primary diagnosis of individuals treated for illicit drug use in the TEDS database. The incidence figures noted in adolescents suggest that it will remain so for some time.

An increase in prescription opiate use and treatment for opiate abuse have been recorded in the drug use and treatment system databases. There are more prescription opiate users than heroin users (and more women than men are prescription opiate users who seek treatment). This is reflected in the fact that more opiate users than heroin users reported getting treatment for their dependence [73]. A different locus of use has been noted for prescription opiate users; that is, the greatest increase has been in rural areas.

Consequently, the treatment delivery system for opiate users is undergoing some unique challenges. Even in light of a worsening situation, funding for pharmacotherapy seems to be diminishing in the publicly funded part of the treatment system. This situation needs to be reversed. Additionally, treatment delivery must be given in reasonable proximity to the users' domiciles. This means that the treatment system must undergo a major change to reach the prescription opiate users who are concentrated in rural areas. The current availability of SUBUTEX and SUBOXONE should help to address the problem of treatment delivery outside urban areas by qualified physicians who can now prescribe these medications as part of their primary-care practices. Physicians are currently constrained to a 30-patient limit. This limit can be changed by legislation or administrative action at the level of the secretary of the U.S. Department of Health and Human Services (DHHS).

The use of methadone for "office-based practice" would be another useful tool for physicians to be able to prescribe for patients. This has been evaluated in several studies and is a feasible treatment approach.

The development and marketing of new medications, primarily nonopiate in nature, are feasible, although the time frame is several years away. Multiple medications of diverse mechanisms might be used as stand-alone medications or as combinations. For example, the testing of a CB-1 antagonist or inverse agonist would be a high priority for testing. Nonopiate medications with good efficacy would usher in another change to the management of opiate dependence. Moreover, these medications would likely enjoy broad societal support.

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NEURONAL PATHWAYS FOR PAIN PROCESSING

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20.1	Introduction	709
20.2	Pain Pathways	711
20.3	Descending Pain Modulatory Pathways	712
20.4	Neuropharmacology of Pain	714
20.5	Localization of Drug Action	718
20.6	Anatomical Drug Interactions and Pain	718
20.7	Conclusion	721
	References	721

20.1 INTRODUCTION

Pain remains a prominent factor in all facets of medicine. Pain helps maintain the safety and integrity of subjects by alerting them to potential injury. Pathways that transmit nociceptive stimuli from the periphery to the central nervous system have been studied extensively. However, situations exist where survival may depend upon minimizing or ignoring pain. A major function of the sensory nervous system is its ability to filter sensory input. The vast array of sensation from all modalities makes it essential for the individual to focus upon a selected few and to place others in the background. Thus, it is not surprising that the nervous system has developed a highly complex system to “filter,” or modulate, the perception of pain.

Pain is unlike most sensations. The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [1]. Thus, in addition to the actual perception of the stimulus, pain requires a processing of this input to provoke a sensation that is perceived as unpleasant. This contrasts with traditional sensations that are more easily quantified, such as touch and

temperature. What makes the understanding of pain particularly difficult is that stimuli that are considered painful in one situation may not be perceived as painful in a different context. Many of us have found bruises after playing a sport without remembering when the injury occurred. Yet, at other times, a less intense injury may be associated with profound pain. The ability to dissociate the stimulus from pain has been observed in patients on opioids, which diminish pain without interfering with more basic sensations. Indeed, patients on opioids may comment, "The pain is still there but it doesn't hurt." Another complexity in understanding pain is its many different types, as illustrated by the wide range of descriptors, such as sharp, dull, aching, shooting, throbbing, and burning.

Clinically, pain has been categorized as somatic, visceral, and neuropathic [1a]. Somatic, or "nociceptive," pain is typically a result of tissue injury. It is common, easily recognized by most people, and typically described as sharp or aching. Of the various types of pain, this is the most sensitive to opioids and the simplest to treat. Visceral pain, as its name implies, involves internal organs. The nature of the pain is dependent upon the structure involved and can feel like cramping, or pressure, such as cholecystitis, pancreatitis, or angina. It can be referred to a different region of the body, such as the shoulder pain seen with diaphragmatic irritation.

Neuropathic pain, on the other hand, is quite novel and unique. It is the most atypical pain and the most difficult to treat. It is usually associated with injury to peripheral nerves, as seen in plexopathies or neuropathies, central pain pathways, as in thalamic pain syndromes, or combinations of both. The pain is typically described as burning, dysesthetic, or shooting, and patients commonly have difficulty describing it. A number of syndromes such as reflex sympathetic dystrophy and causalgia (complex regional pain syndromes) are due to involvement of the sympathetic nervous system. Although traditional analgesics, including the opioids, are used to treat this pain, they often require far higher doses than normal, which in turn can lead to increased side effects and difficulties with the patients tolerating them. Many other classes of drugs are used to manage neuropathic pain, including antidepressants and anticonvulsants. Unfortunately, it is not unusual for these drugs, either alone or in combination, to give only partial relief.

In addition to its quality, pain is also characterized by its duration as either acute or chronic. Once a pain has been present for six months, it is typically considered chronic [1a]. Clinically, acute and chronic pain can be separated by more than their duration. Acute pain is often associated with autonomic effects, such as tachycardia, papillary dilation, and diaphoresis, signs typically associated with pain. However, most of the autonomic signs seen acutely are lost in chronic-pain states, making it more difficult for the clinician to assess the severity of the pain. Indeed, with chronic pain the clinician is left with only the assessment by the patient. Chronic pain is further complicated by a number of factors and can be very difficult to treat. These include sensitization and "wind-up" and the commonly associated depression seen in these patients. Other issues, such as the impact of potential disability and financial issues, also can complicate its diagnosis and treatment.

Thus, pain is not simple. It requires higher integrative processing to define its unpleasant aspects and its perception is highly dependent upon the situation in which it occurs and its meaning. There are three major types of pain that differ in their sensitivities to pharmacological approaches, and most clinical pain is composed of combinations of them.

20.2 PAIN PATHWAYS

Pain pathways are complex. They involve far more than the simple conduction of an impulse from a peripheral nociceptor to the brain. Ascending systems dissociate discriminative aspects of pain from the emotional components (Table 20.1) while descending pathways provide a mechanism for the modulation of nociceptive transmission at all levels of the neuroaxis. Discriminative aspects of pain have been well localized to specific pathways and regions of sensory cortex. In contrast, the affective component is mediated predominantly through subcortical pathways involving limbic circuits, making their mapping difficult.

Nociceptors have a widespread localization peripherally in skin and subcutaneous tissues, joints, muscles, blood vessels, and viscera and are located on neurons whose cell bodies are within the dorsal root or trigeminal ganglia [1a–4]. Myelinated nociceptors respond to either intense mechanical or mechanothermal stimuli whereas unmyelinated nociceptors are polymodal, responding to thermal, mechanical, or chemical stimuli. Certain unmyelinated nociceptors are particularly sensitive to pH and chemical agents, such as histamine and bradykinin, which are released by tissue damage and can be further sensitized by serotonin and prostaglandins in the damaged tissues.

Aδ fibers and C fibers carry pain information from the periphery to the central nervous system. Aδ fibers are myelinated and thicker than the unmyelinated C fibers, explaining their faster transmission of pain impulses (5–30 m/s when compared to C fibers (0.5–2 m/s) and contributing to the phenomenon of “first” and “second” pain.

The primary sensory neuron cell bodies are located in the dorsal root ganglia or cranial nerve ganglia. Axons from dorsal root ganglia enter the spinal cord through the dorsal horn where they ascend or descend for several spinal cord segments before interacting with intrinsic spinal cord neurons. Aδ fibers typically terminate within lamina I (marginal zone) and, to a lesser extent, within lamina V. C fibers tend to

TABLE 20.1 Comparison of Neospinothalamic and Paleospinothalamic Pain Pathways

	Neospinothalamic Tract	Paleospinothalamic Tract
Anatomy		
Thalamic target	VPL	Intralaminar and midline nuclei
Intermediate synapses between dorsal horn and thalamus	None	Periaqueductal gray, reticular formation
Thalamic projections	Somatosensory cortex (parietal lobe)	Subcortical (cingulate gyrus, limbic system, hypothalamus)
Pain sensation	Fast or first pain Discriminative component (intensity, localization, quality) Well localized	Slow or second pain Affective component (“hurt”) Poorly localized
Other sensations	Light touch, temperature	

Note: Sensory neurons with their cell bodies in the dorsal root ganglia enter the dorsal horn of the spinal cord where the Aδ fibers synapse in laminae I and V and ascend to the thalamus as the neospinothalamic tract and the C fibers synapse primarily in laminae I and II and ascend as the paleospinothalamic, or spinoreticular, tract.

project to lamina I and lamina II (substantia gelatinosa) where they synapse with interneurons and neurons that project rostrally. Some axons synapse on motor neurons in the ventral horn, completing the pain reflex arc. Trigeminal ganglia axons enter the brain stem and project to the spinal nucleus of the trigeminal tract, which is analogous to the substantia gelatinosa within the spinal cord (Fig. 20.1b).

Nociception is transmitted rostrally through two important pathways: the lateral spinothalamic tract (Fig. 20.1, Table 20.1) and the anterior spinothalamic tract. The lateral spinothalamic tract receives information regarding the location and intensity of the nociceptive stimuli and has a distinct somatotopic organization within the tract as it ascends and comprises the neospinothalamic pathway. Axons in the tract cross to the opposite side of the spinal cord over several spinal segments through the anterior commissure and proceed to ascend to the ventroposterior lateral (VPL) nucleus of the thalamus. Axons in the trigeminal thalamic tract cross the midline and synapse in the ventroposterior medial (VPM) nucleus of the thalamus. From the thalamus, these tracts ascend to primary somatosensory cortex with its well-defined somatotopic organization. Cortical lesions, such as seen in strokes, lead to loss of this well-localized pain sensation. This pathway transmits primarily nociception carried by A δ fibers and corresponds to first pain, in distinction from second pain, which involves primarily C fibers and the paleospinothalamic tract.

The anterior spinothalamic, which is a component of the paleospinothalamic tract (Fig. 20.2), is involved with the affective, or unpleasant, component of pain. It carries “second” or “slow” pain and it is this component of pain that is sensitive to opioids. Unlike the neospinothalamic tract which ascends directly to the thalamus, the paleospinothalamic tract has numerous interactions with a range of brain stem nuclei (nucleus gigantocellularis and parabrachial region), the reticular formation, and periaqueductal gray before converging on the medial and intralaminar nuclei of the thalamus. From there, the impulses are relayed not to the somatosensory cortex but to limbic structures and association cortex instead, including the anterior cingulate gyrus, amygdala, and hypothalamus. The importance of many of these higher structures in pain clinically has now been supported by positron emission tomography [5, 6]. Nociception carried by this pathway is slower, due to both the slower conduction rate of the C fibers and the multiple synapses within the pathway as it ascends, leading to the description of this pain as second or slow pain. Unlike the rapidly conducted first pain, second pain is poorly localized and more diffuse with a dysethetic quality. The selectivity of opioids for this pathway may help explain the observation by some patients after taking opioids that they can still feel the pain, but it does not bother them, consistent with a selectivity of the opioid response to the affective component of pain, or “hurt,” carried by the paleospinothalamic tract.

20.3 DESCENDING PAIN MODULATORY PATHWAYS

The central nervous system can modulate the perception of pain. This can be observed in a variety of situations, such as athletes who are unaware of an injury until after the game or the lower morphine requirements of wounded soldiers during the stress of battle [7]. Even acupuncture has been proposed to involve the activation of intrinsic antinociceptive pathways [8]. In the 1960s, Wall and Melzack proposed

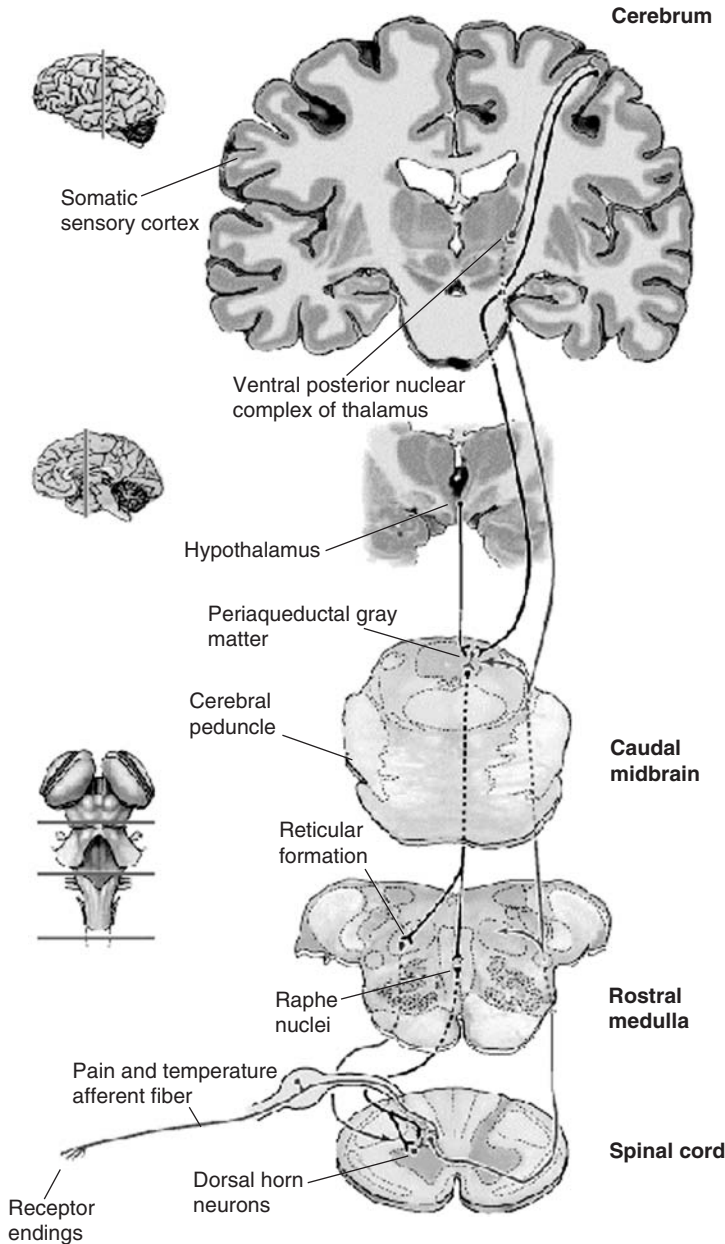


Figure 20.1 Ascending spinothalamic tract. Major pathways for pain (and temperature) sensation. (a) Spinothalamic system. (b) Trigeminal pain and temperature system, which carries information about these sensations from the face. (Reproduced with permission from D. Purves et al. (2001). *NeuroScience*. Sinauer, Sunderland, MA, Fig. 10.3.) (See color insert.)

the “gate control” theory, which has now been much expanded, to explain how central systems can modulate peripheral nociceptive input [9]. Descending pathways from the brain that are heavily influenced by opioids and a number of other selected neurotransmitters are primarily responsible for these actions (Fig. 20.3) [2, 10–12].

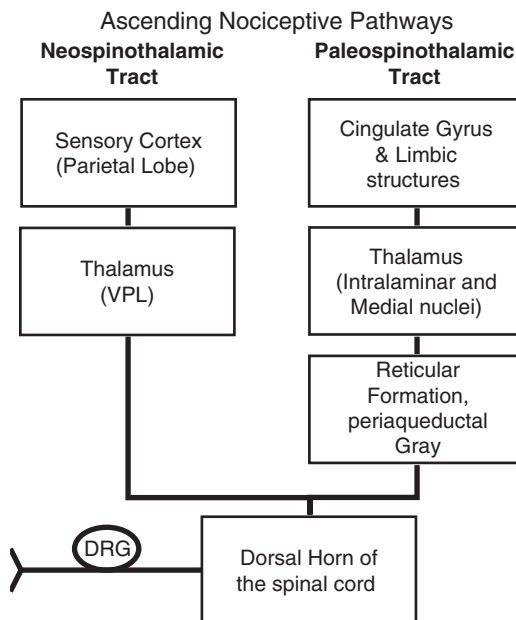


Figure 20.2 Ascending nociceptive pathways.

Pain sensation is modulated by the descending pain modulatory pathways in a balance of excitation and inhibition [2, 3, 13, 14]. The periaqueductal gray (PAG) is particularly important in this system. Early studies showed that morphine was an extremely potent analgesic when microinjected into this site [15]. Additional studies have identified a number of midbrain areas, including the locus ceruleus, nucleus raphe magnus, and nucleus paragigantocellularis [11, 16–18]. Stimulation of the PAG produced analgesia in rodents that was naloxone reversible [19–21], observations similar to those seen clinically [22]. These studies imply that activation of the PAG would produce an opioidlike analgesia. The PAG receives input from a wide range of structures, including the amygdala, frontal and insular cortex, and hypothalamus. It projects to the nucleus raphe magnus, which then descends to suppress pain transmission in the dorsal horn of the spinal cord. The PAG receives input from a wide range of structures, including the somatosensory cortex, frontal and insular cortex, amygdala, and hypothalamus. The PAG, in turn, projects to neurons in the nucleus raphe magnus and other nuclei in the rostral ventral medulla, the nucleus reticularis paragigantocellularis in particular [6–9]. The medullary nuclei transmit signals in the dorsolateral column down to the same regions of the dorsal horn of the spinal cord in which primary sensory neurons synapse onto secondary sensory neurons. Descending axons from the PAG, raphe nuclei, and locus ceruleus also project directly to the spinal cord.

20.4 NEUROPHARMACOLOGY OF PAIN

Many neurotransmitter systems have been implicated in both the transmission of nociception and its modulation. Indeed, few transmitters are not involved. Some

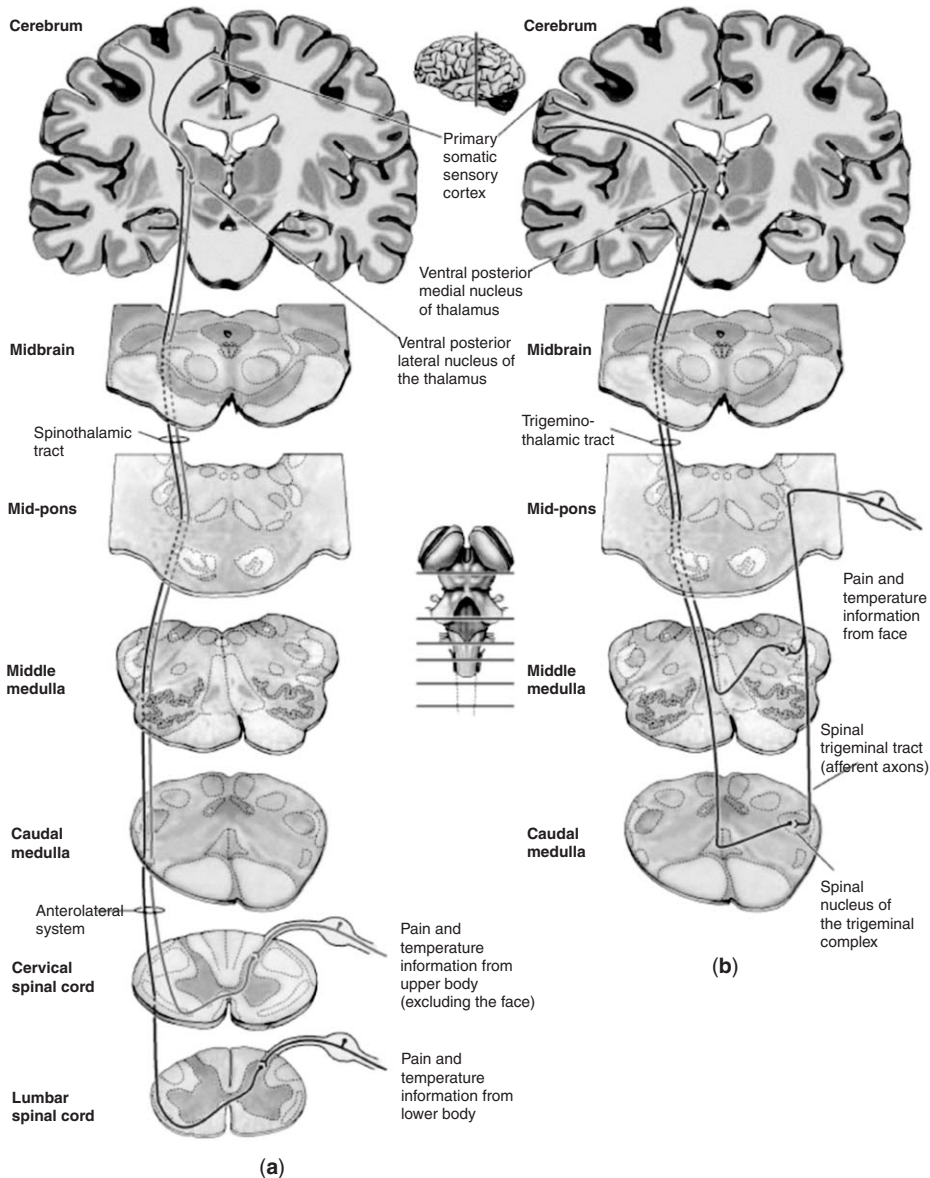


Figure 20.3 Descending pain modulatory pathways. The descending systems that modulate the transmission of ascending pain signals. These modulatory systems originate in the somatic sensory cortex, the hypothalamus, the periaqueductal gray matter of the midbrain, the raphe nuclei, and other nuclei of the rostral ventral medulla. Complex modulatory effects occur at each of these sites, as well as in the dorsal horn. (Reproduced with permission from D. Purues et al. (2001). *NeuroScience*. Sinauer, Sunderland, MA, Fig. 10.5). (See color insert.)

provide potent pain relief, such as the endogenous opioids [1a], nicotinic analogs [23], and cannabinoids [24, 25], while others demonstrate limited activity due to ceiling effects or are restricted to specific pain syndromes. One of the best examples of agents with ceiling effects are the nonsteroidal anti-inflammatory drugs, which are quite

effective for mild/moderate pain but lack the efficacy needed for severe pain. Antidepressants and anticonvulsants are typically used for neuropathic pain, such as the peripheral neuropathies and postherpetic neuralgia, or headaches. Trigeminal neuralgia with its lancinating pain is quite unique and the first-line drug to treat this relatively common disorder is the anticonvulsant carbamazepine. Thus, from the clinical perspective, a wide range of drugs are valuable in pain management, with various types of pain responding differently to these agents [1a, 3, 26–28].

The most effective clinical agents in pain management remain the opioids. These drugs mimic the actions of the endogenous opioid peptides and have been classified by their selectivity for the three major classes of receptors: μ , δ , and κ . Although some agents with κ actions are available clinically, most opioid analgesics are morphine-like and are selective for μ -opioid receptors. Extensive structure–activity studies have dissected the structure of morphine and generated a host of drugs with widely dissimilar structures that act through μ receptors (Fig. 20.4). Although δ compounds are effective in animal pain models, none have yet been approved for general clinical use. There are three major families of endogenous opioids, each with its own precursor peptide: enkephalins, dynorphin A, and β -endorphin [29, 30]. The enkephalins and dynorphin A are the endogenous ligands for the δ and κ_1 receptors, respectively. The endogenous ligand for the μ receptors is still uncertain. There is evidence implicating endomorphin 1 and endomorphin 2 as well as β -endorphin, but some questions still remain. The complexity of the μ -opioid system is further illustrated by the recent identification of a number of splice variants of the cloned μ -opioid receptor MOR-1 with their distinct regional distributions and pharmacological properties [31–36]. The question that now arises is whether or not the identification of these splice variants of MOR-1 will enable the dissociation of analgesia from its troublesome side effects.

As the number of neurotransmitters involved with pain pathways has expanded, they have raised the possibility of novel approaches toward pain management [37]. Many peptidergic systems were considered “anti-opioid,” including cholecystokinin (CCK), neuropeptide FF (NPFF), and melanocyte-inhibiting factor (MIF)-related peptides, although their actions are complex and can inhibit or enhance nociceptive perceptions depending upon the situation [38]. The antio-pioid activity of CCK appears to involve activation of CCK_B receptors, while stimulation of CCK_A receptors can induce an opioid-like activity. The major focus of CCK has been on its modulation of opioid tolerance [39, 40], but this approach has not proven successful clinically. The role of substance P and calcitonin gene-related peptide (CGRP) in nociceptive transmission in the spinal cord has been well established. Opioids inhibit their release at the spinal level and antagonists looked promising in animal models. Yet, substance P antagonists have not proven themselves clinically [41].

More traditional neurotransmitter systems implicated in pain mechanisms that may be of therapeutic importance include the monoamines, acetylcholine, glutamate, glycine, and both sodium and calcium channels. Almost all the monoamines can be implicated in pain modulation, perhaps explaining the importance of many of the antidepressants in pain management. Both muscarinic and nicotinic acetylcholine systems influence pain. Many of the antidepressants used clinically are effective muscarinic antagonists, which may help explain a portion of their utility. A highly potent nicotinic acetylcholine receptor agonists has been developed [23], but its side effects were too problematic for generalized use.

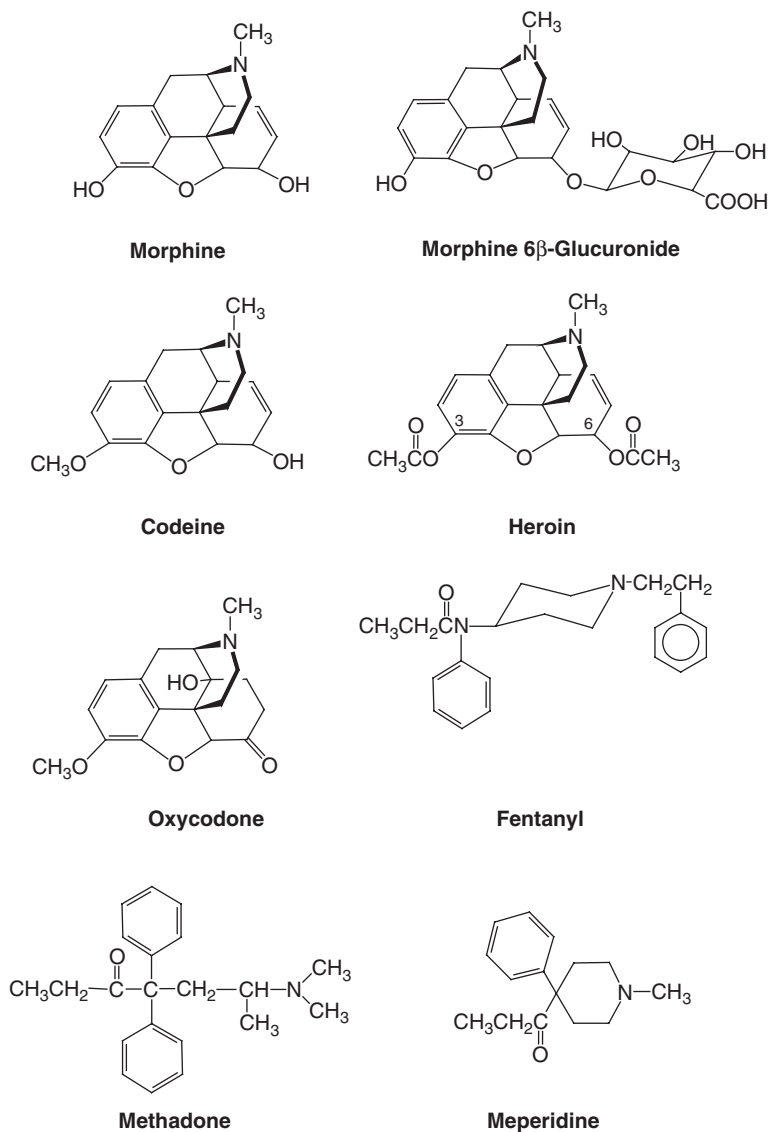


Figure 20.4 Structures of selected opioids.

Glutamate has a special place in pain pharmacology [42–44]. Although *N*-methyl-D-aspartate (NMDA) receptors are involved in the production of opioid tolerance, their role in central sensitization and wind-up may be more important. These systems lead to the facilitation of the transmission of pain impulses and may play a role in the development of chronic pain states, which can be very difficult to treat. This has led some clinicians to use “preemptive analgesia” to prevent sensitization. Unfortunately, the side-effect profile of NMDA antagonists is problematic, making the use of these agents clinically unacceptable with the exception of memantine.

20.5 LOCALIZATION OF DRUG ACTION

Peripheral mechanisms associated with pain have been extensively explored. Local anesthetics, which block sodium channels, effectively prevent pain [4], but they suffer from their lack of selectivity in that they also block other sensory modalities, such as light touch. A variety of transmitter receptors located on peripheral nerves also can modulate pain transmission. For example, opiate receptors have now been identified on sensory dorsal root ganglion neurons both in the periphery and presynaptically in the dorsal horn of the spinal cord [45–49]. Opioids traditionally were thought to act centrally by presynaptic inhibition of the release of nociceptive transmitters, such as substance P or CGRP, as well as on intrinsic spinal neurons. One of the MOR-1 variants, MOR-1C, is colocalized presynaptically on neurons containing CGRP while the predominant MOR-1 is not [51]. The release of CGRP can be blocked by morphine, presumably through these MOR-1C receptors. Substance P neurons terminating within the dorsal horn do appear to be associated with MOR-1 [51]. Opioids inhibit the release of substance P, diminishing nociceptive transmission [52]. Substance P drugs also can modulate pain perception at the spinal cord level, but their utility clinically is not clear [41, 53].

Opioid receptors have been demonstrated on peripheral nerves, explaining the analgesic actions of opioids administered locally to the nerves and without central activity [54–60]. Similarly, other drugs acting through various neurotransmitter systems also can directly influence peripheral nerves, including the antidepressant amitriptyline, the NMDA antagonist ketamine, and a variety of channel blockers, as well as through central sites of action [60].

Within the brain the sites involved with nociception include a broad range of structures, particularly regions within the brain stem and limbic systems. Our understanding of the regions involved initially came from classical studies examining the sensitivity of various brain structures to the microinjection of opioids [15]. The primary sites implicated in morphine analgesia included the PAG, the nucleus raphe magnus, and the locus ceruleus, regions subsequently shown to contain high densities of opioid receptors both autoradiographically [61–64] and immunohistochemically and at the messenger RNA (mRNA) levels [65–67].

Chronic pain is pharmacologically distinct from acute pain and involves plasticity with neurochemical changes. The maintenance of chronic pain has been associated with activation of glutamate receptors. NMDA mechanisms act centrally in sensitization and are involved with plasticity. Indeed, the identification of NMDA receptors in “windup” or central sensitization [68, 69] has led to the concept that early and aggressive treatment of pain can help minimize the sensitization process [70].

20.6 ANATOMICAL DRUG INTERACTIONS AND PAIN

Pain pathways have an additional complexity. For example, morphine is a potent analgesic when administered either supraspinally or spinally in the rat. However, administering morphine to both sites simultaneously leads to a profound potentiation of their actions (i.e., synergy) (Table 20.2) [71]. The total dose of morphine required for an analgesic response when it is given both spinally and supraspinally is

TABLE 20.2 Spinal–Supraspinal Morphine Interactions

Site of Drug Administration	Morphine ED ₅₀ (μg)
Supraspinal	10 (7.23–14.17) i.c.v.
Spinal	4.2 (3.1–5.4) i.t.
Combination ^a (supraspinal–spinal)	0.7 (0.38–1.08) (0.35 i.c.v. + 0.35 i.t.)

Note: ED₅₀ = median effective dose; i.c.v. = intracerebrovascular; i.t. = intrathecal.

^aThis value corresponds to the ED₅₀ for the total dose for the animal using a supraspinal–spinal ratio of 1:1. Thus, the ED₅₀ for the combination corresponds to 0.35 μg given in each location. From [71].

at least sixfold lower than if it is given to either location alone. Thus, there are important interactions among the sites along the pain modulatory pathways.

Since then, other examples of site–site synergy have been observed. Morphine is an active analgesic when administered into the PAG, rostroventral medulla (RVM), or locus ceruleus. However, coadministration of low morphine doses which are inactive alone into combinations of these three regions elicits dramatic analgesic responses, implying the existence of synergy. The most effective combination is the PAG–RVM, whereas the PAG–LC and RVM–LC combinations are much less efficacious. Thus, there are important brain stem interactions involved with morphine analgesia.

Peripherally acting opiates also synergize with central sites. Topical morphine is effective in traditional analgesic paradigms in mice and rats [54, 58, 59, 72–74]. Under conditions that are associated with no appreciable systemic absorption, a variety of opioids produce reproducible dose–response relationships and show the same pharmacological characteristics as seen centrally. However, the combination of topical and spinal morphine is highly synergistic (Fig. 20.5). Thus, morphine shows interactions among a wide range of sites within the central and peripheral nervous systems, adding to the complexity of the system.

These interactions among sites help explain some clinical observations. Epidural opiates are widely used in pain management. The advantage is that there are far fewer side effects, such as nausea, constipation, and respiratory depression, and the drugs can give prolonged pain relief. When given epidurally, morphine concentrations in the cerebrospinal fluid at the spinal level reach levels far beyond those achieved following systemic administration. Equally important, there also is significant systemic absorption through Batson's plexus, leading to blood levels not far below those seen following intramuscular administration. Studies in animals have shown that very low morphine concentrations at the spinal level can markedly potentiate systemic morphine (Table 20.3). Intrathecal doses of morphine that are approximately 10-fold below the ED₅₀ of spinal morphine can shift the systemic morphine dose response curve sixfold to the left. Thus, the utility of epidural morphine likely reflects the combined actions of spinal and systemic drug. Since the side effects associated with morphine, such as sedation, nausea, and respiratory depression, are mediated supraspinally, side effects are avoided by the low levels of systemic drug.

The importance of peripheral sites of action have also been established in other situations. When morphine is given centrally into the lateral ventricle, one would

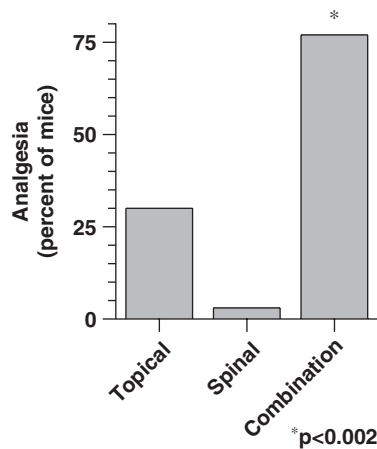


Figure 20.5 Interactions between topical and spinal morphine. Groups of mice ($n<10$) received topical morphine (15 mM, 2min) alone or with spinal (100 ng, i.t.). The spinal morphine dose alone had no observable analgesic action. After 30 min, when the response to topical drug alone was lost, the responses of the combinations were significantly greater ($p<0.002$). From [59].

TABLE 20.3 Peripheral–Central Morphine Interactions

Route of Administration	ED ₅₀	Shift
Spinal (i.t.) alone	305 ng, i.t.	
Systemic alone	3.1 mg/kg, s.c.	
+ 25 ng, i.t.	0.5 mg/kg, s.c.	6
+ 50 ng, i.t.	0.3 mg/kg, s.c.	10
+ 200 ng, i.t.	0.04 mg/kg, s.c.	84

Mice received various doses of morphine intrathecally to determine the ED₅₀. Additional groups of mice then received the stated fixed dose of intrathecal morphine and various doses of morphine [subcutaneous (s.c.)]. The ED₅₀ for the systemic morphine was then determined. From [54].

have assumed that its actions were restricted to the brain. However, morphine given intrathecally is rapidly secreted from the brain into the periphery by mechanisms involving P glycoprotein and can be detected in the blood [75]. These systemic levels of morphine are relevant, since a topical antagonist limited to the periphery shifts the analgesic response of supraspinal morphine over fourfold, implying that the overall response reflected interactions between central and peripheral sites. Downregulation of P glycoprotein using either an antisense or a knockout approach eliminates the ability of the topical antagonist to shift the supraspinal analgesic response, consistent with the role of P glycoprotein in transporting the drug to the systemic circulation. Thus, site–site interactions are extremely important in understanding the activity of analgesic drugs.

Interactions have also been seen among different groups of drugs working through different mechanisms. This is best illustrated by the interactions between local anesthetics and opioids at the spinal level [76] and peripherally [77, 78]. Similar

interactions topically have been observed with combinations of antidepressants, NMDA antagonists, and other classes of drugs [60].

20.7 CONCLUSION

The pathways transmitting nociceptive stimuli and those involved with its modulation are exceedingly complex. Interactions between these counteracting systems occur at multiple levels of the neuraxis, with each influencing the others. While the anatomy of the pathways has been known for many years, understanding their functional and pharmacological significance is more recent. These pathways are not static “transmission lines.” Rather, they are interacting pathways providing complex pharmacological interactions that may prove helpful in our continuing efforts in pain management.

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VANILLOID RECEPTOR PATHWAYS

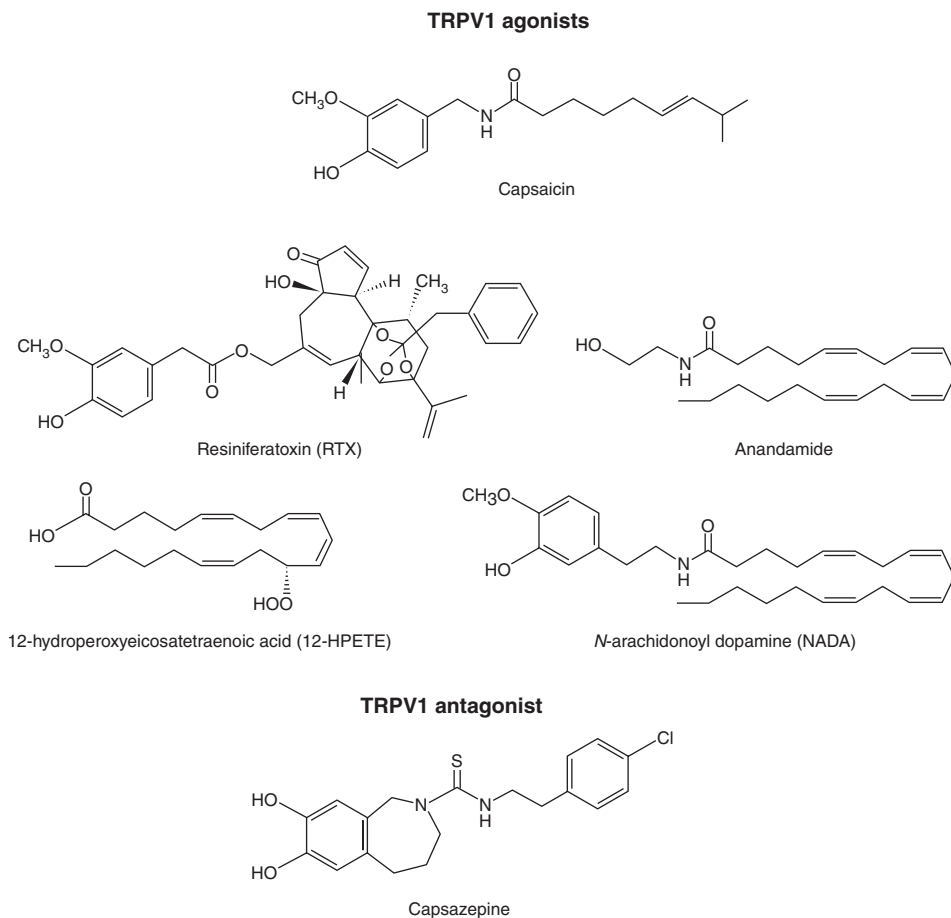
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21.1	Cloning of Vanilloid Receptor	727
21.2	TRPV1 Exhibits Highly Specific Expression Pattern	729
21.3	TRPV1 Activation by Capsaicin, Protons, and Heat	730
21.4	Diverse Chemical Activators of TRPV1	732
21.5	Antagonists of TRPV1 and Their Implication in Clinical Settings	733
21.6	Sensitization of TRPV1	733
21.7	Desensitization of TRPV1	735
21.8	Knockout Mouse Study	736
21.9	Other TRPV Channels Involved in Nociception	736
	References	737

21.1 CLONING OF VANILLOID RECEPTOR

Hot chili peppers have been long used in food, and people know well that the peppers produce a burning sensation in the mouth. The structure of capsaicin, a main pungent ingredient of capsicum peppers, was solved as 8-methyl-*N*-vanillyl-6-nonenamide in 1928 [1], and several decades later Hungarian researchers revealed that capsaicin could cause pain and desensitize further capsaicin challenge at the same time in rats [2, 3]. Three important findings—(1) that capsaicin and resiniferatoxin (RTX), a pungent substance derived from *Euphorbia* species, share a similar structure, including a vanilloid motif necessary for pungency (Fig. 21.1) [4]; (2) specific bindings of [³H]RTX to the small-diameter sensory neurons [5]; and (3) development of a competitive antagonist of vanilloid action, capsazepine (Fig. 21.1) [6]—resulted in the belief that a receptor protein was responsible for the capsaicin action. Data obtained using electrophysiological methods provided further evidence that vanilloid receptor activation allows cation influx through its ionic pore, leading to the depolarization of the nociceptive neurons, followed by action potential generation. This seems to be one of the mechanisms whereby noxious stimuli are



renamed TRPV1 as the first member of the TRPV (vanilloid) subfamily. A prototypical member of the TRP superfamily of ion channels was reported in 1989 and found to be deficient in a *Drosophila* mutant exhibiting abnormal responsiveness to continuous light [9]. Now, this large TRP superfamily of ion channels is divided into seven subfamilies (TRPC, TRPV, TRPM, TRPP, TRPN, TRPA, and TRPML) [10].

21.2 TRPV1 EXHIBITS HIGHLY SPECIFIC EXPRESSION PATTERN

TRPV1 expression at both messenger RNA (mRNA) and protein levels was extensively examined, and those studies revealed that TRPV1 is highly expressed in dorsal root, trigeminal, and nodose ganglia, specifically within a subset of small- to medium-diameter sensory neurons that project to the superficial layers of the spinal cord (laminae I and II), trigeminal nucleus, and solitary tract nucleus, respectively [8, 11–14]. These observations, plus the fact that the TRPV1 protein is detected in nerve terminals of the bladder [14], indicates that TRPV1 is expressed in both central and peripheral termini of sensory neurons involved in nociception. This expression pattern is consistent with the finding that capsaicin selectively activates unmyelinated C fibers and thinly myelinated A δ fibers. Although TRPV1 expression is also reported in a number of other neuronal and nonneuronal tissues such as brain, keratinocytes, and urinary epithelial cells [15–17] where nonnociceptive functions are hypothesized, expression level in sensory neurons appears to be much higher than in any other region. Primary afferent nociceptors have been histochemically divided into two distinct classes in the adult rodent: one expresses neuropeptides such as SP and CGRP; the other expresses specific enzyme markers such as fluoride-resistant acid phosphatase and binds the isolectin By (IBy) [18]. These two classes of neurons are sensitive to the neurotrophic nerve growth factor (NGF) and glial cell line–derived neurotrophic factor (GDNF), respectively. Both subpopulations of sensory neurons respond to capsaicin, and colocalization studies of TRPV1 with IB4 and SP probes revealed that many SP immunoreactive cells or IB4-positive cells costained with TRPV1, although $\sim 10\%$ of the TRPV1-positive neurons did not stain with either SP or IB4 [14].

It has been reported that inflammation or tissue injury induced an increase in the number of unmyelinated C fibers expressing TRPV1 [19]. Furthermore, increased TRPV1 expression induced by inflammation was predominantly observed in myelinated A δ fibers compared to C fibers [20]. NGF induced increase of TRPV1 mRNA and release of CGRP with capsaicin treatment in primary cultured DRG neurons [21]. Activation of p38MAPK (mitogen-activated protein kinase) by NGF was found to enhance the translocation of TRPV1 proteins from cell bodies in DRG to sensory nerve endings [22]. Furthermore, p38MAPK inhibitor reduced inflammatory hyperalgesia. Thus, increase of TRPV1 expression in the sensory nerve endings seems to be involved in the development of hyperalgesia.

In the visceral organs, including intestine, TRPV1 was found to be expressed throughout the sensory neurons, and most apparent expression was observed in nerve fibers that innervated the myenteric plexus of visceral organs [23]. TRPV1-expressing neurons detected with specific anti-TRPV1 antibody seem to be not vagal afferent fibers but spinal in origin.

21.3 TRPV1 ACTIVATION BY CAPSAICIN, PROTONS, AND HEAT

Upon exposure to capsaicin, TRPV1 exhibits an outwardly rectifying, nonselective cation current with high Ca^{2+} permeability [8]. Upon application of capsaicin to membrane patches excised from human embryonic kidney (HEK) cells expressing TRPV1, clear single-channel openings were observed (conductance of ~ 80 pS for Na^+) (Fig. 21.2), indicating that no cytosolic second messengers are necessary for TRPV1 activation. This channel also exhibits a voltage-dependent gating property such that depolarization promotes TRPV1 activation [24]. TRPV1 expressed alone in HEK293 cells or *Xenopus* oocytes can account for the majority of the electrophysiological properties exhibited by native capsaicin receptors in sensory neurons, including ligand affinity, permeability sequence, current–voltage (I – V) relationship, conductance, and open probability at both single-channel and whole-cell levels [8, 14, 24, 25]. These results suggest either that TRPV1 can form homomultimers (probably tetramer) without other subunits or that incorporation of subunits other than TRPV1 does not influence the functional properties.

Because capsaicin and its analogs such as RTX are lipophilic, it is quite possible that they pass through the cell membrane and act on binding sites present in the intracellular surface of TRPV1. An apparent time lag between capsaicin uptake and pungent sensation might be partially explained by such a process. Comparison of rat TRPV1 with its avian ortholog from chicken sensory neurons, which is insensitive to capsaicin, together with mutational analysis revealed that tyrosine 511 and serine 512, located at the transition between the second intracellular loop and the third TM domain, might interact with vanilloid ligands at the intracellular face of the membrane [26].

Tissue acidification is induced in pathological conditions such as ischemia and inflammation. Such acidification exacerbates or causes pain [27, 28]. Acidification of the extracellular milieu has two primary effects on TRPV1 function [14]. First, extracellular protons increase the potency of heat or capsaicin as TRPV1 agonists, in part, by lowering the threshold for channel activation by either stimulus. Second, extracellular protons can, themselves, be viewed as agonists because further acidification (to $\text{pH} < 6.0$) leads to channel opening at room temperature. Acidic solution evoked ionic currents with a mean effective concentration (EC_{50}) of about $\text{pH} 5.4$ at room temperature when applied to outside-out but not inside-out membrane patches

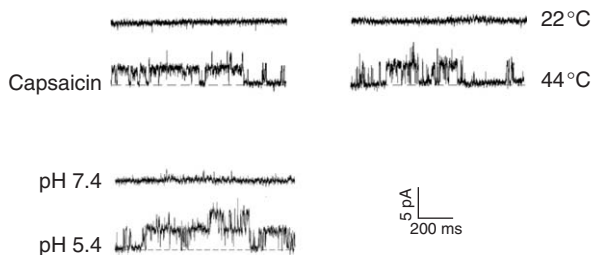


Figure 21.2 Representative single-channel currents in membrane patches excised from HEK293 cells expressing TRPV1 to bath-applied capsaicin ($100\ \mu\text{M}$, inside out), protons ($\text{pH} 5.4$, outside out), or heat (44°C , inside out). Dotted lines indicate closed level. Holding potential $+40$ mV.

excised from HEK293 cells expressing TRPV1 (Fig. 21.2), suggesting that protons act on amino acids in the extracellular domain of TRPV1 having side chain pK_a values in the physiologically relevant range. Mutational analyses revealed that glutamate 600, located within a putative extracellular domain, serves as an important regulator site for proton potentiation of TRPV1 activity, whereas glutamate 648 is involved in direct proton-evoked activation of TRPV1 [29]. In sensory neurons, proton-evoked currents consist of two major components: one is rapidly inactivating and Na^+ selective with a linear $I-V$ relation [30]; the other is a more sustained, nonselective cation conductance with an outwardly rectifying $I-V$ profile [31]. The latter is believed to underlie the prolonged sensation of pain, and TRPV1 may represent a responsible molecular entity for this component. In addition to its activating or modulating effects on TRPV1, protons were found to permeate the nonselective TRPV1 pore in acidic extracellular solution, resulting in marked intracellular acidification [32].

The burning quality of capsaicin-induced pain suggests that capsaicin and heat may evoke painful responses through a common molecular pathway. TRPV1 was, in fact, found to be activated by heat at $>43^\circ C$ (Fig. 21.3), a temperature threshold

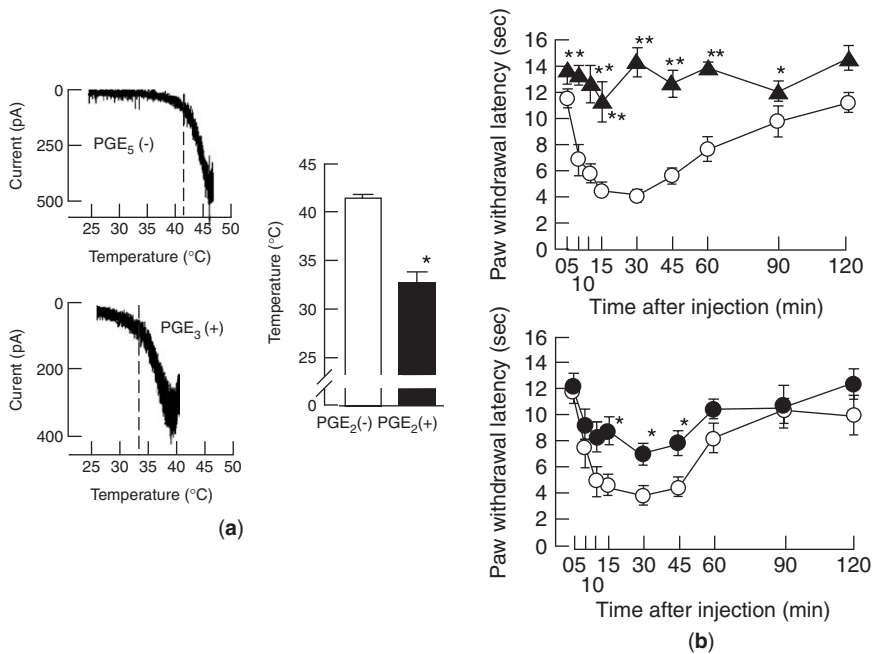


Figure 21.3 Reduction of temperature threshold for TRPV1 activation with PGE_2 ($1\mu M$), and inhibition of PGE_2 -induced thermal hyperalgesia in TRPV1- or EP_1 -deficient mice. (a) Representative temperature response profiles in the absence (upper) and presence (lower) of PGE_2 (left). Temperature threshold for TRPV1 activation in the presence of PGE_2 ($30.6 \pm 1.1^\circ C$) was significantly lower than that in the absence of PGE_2 ($40.7 \pm 0.3^\circ C$) (right). (*) $p < 0.05$ vs. $PGE_2 (-)$. (b) Paw withdrawal latency after injection of PGE_2 into hind paw in wild-type mice (open circle), TRPV1-deficient mice (closed triangle), or EP_1 -deficient mice (closed circle). (*) $p < 0.05$ and (**) $p < 0.01$ vs. wild-type mice. (From Moriyama et al., *Mol. Pain* 1: 3, 2005).

that is similar to that at which heat evokes pain *in vivo*, suggesting that TRPV1 is involved in the detection of painful heat by primary sensory neurons [14]. Heat-evoked TRPV1 currents show properties similar to those of capsaicin-evoked currents. However, there are several differences in the properties, including cationic permeability ratio and Ca^{2+} -independent desensitization, suggesting that TRPV1-mediated responses to capsaicin and heat involve distinct but overlapping mechanisms. Heat-evoked single-channel openings were observed in inside-out membrane patches excised from HEK293 cells expressing TRPV1 (Fig. 21.2), suggesting that TRPV1 is, itself, a heat sensor.

21.4 DIVERSE CHEMICAL ACTIVATORS OF TRPV1

Not only capsaicin and RTX but also other chemically related substances such as zingerol and piperin, chemicals responsible for the pungency of ginger and black pepper, respectively, were reported to have the ability to activate TRPV1 [33]. In addition to these vanilloid compounds, allicin, the chemical causing pungency in garlic, was found to activate TRPV1 [34].

Numerous endogenous lipid metabolites of arachidonic acid (AA) in plasma membrane are also capable of activating TRPV1. Palmitoyl ethanolamide (anandamide) (Fig. 21.1), an endocannabinoid, is the first such substance in lipids which might work as an endogenous ligand for TRPV1 [35]. However, anandamide's ability to be a physiologically relevant activator of TRPV1 is controversial because concentrations at which anandamide can activate TRPV1 (micromolars) are higher than those that activate G-protein-coupled cannabinoid CB_1 or CB_2 receptors (nanomolars) and because anandamide exhibits partial agonism [36]. Various oxygenated AA derivatives, including 12- and 15-hydroperoxyeicosatetraenoic acid (12-HPETE and 15-HPETE, respectively) (Fig. 21.1) were also found to activate TRPV1 [37]. These AA metabolites can be produced by lipoxygenases that introduce molecular oxygen in AA released from the membrane by phospholipase A_2 enzymes. Again, reported EC_{50} values for activation of TRPV1 by 12-HPETE or 15-HPETE are relatively high (micromolars), although, to date, no pharmacological study has been performed to examine how much 12-HPETE or 15-HPETE is produced in pathological conditions. However, Shin et al. provided evidence that 12-HPETE is produced endogenously in sensory neurons upon stimulation of sensory nerve endings by the inflammatory mediator bradykinin and activates TRPV1 [38]. This suggests that lipoxygenase products might be important in the development of inflammatory pain. *N*-Arachidonoyl dopamine (NADA) (Fig. 21.1), originally characterized in the striatum, is a full agonist of TRPV1 and the most potent endogenous lipid ligand discovered to date [39]. *N*-Oleoidopamine also possesses the ability to activate TRPV1 with the same potency as NADA [40]. However, the distribution of NADA in dorsal root ganglia (DRG) is much lower than in brain regions (striatum, hippocampus, and cerebellum). In addition, as yet, no TRPV1-mediated physiological or pathological conditions have been attributed to endogenously formed NADA, making the concept that NADA can produce pain *in vivo* obscure. On the other hand, widespread distribution of the compounds, not only in peripheral tissues innervated by TRPV1-expressing afferents, but also in the spinal cord and brain, might explain why TRPV1 is expressed throughout the central

nervous system, albeit at levels lower than in nociceptive neurons. To qualify as an endogenous activator of TRPV1, the three classes of lipids described above have to be formed by cells and be released in an activity-dependent manner in sufficient amounts to evoke a TRPV1-mediated response. Further experiments should be done to clarify the importance of the lipids.

21.5 ANTAGONISTS OF TRPV1 AND THEIR IMPLICATION IN CLINICAL SETTINGS

The two antagonists that have been traditionally used to block capsaicin receptors are capsazepine and ruthenium red. Capsazepine shares structure similarity with vanilloid compounds (Fig. 21.1). *In vitro*, capsazepine competitively antagonizes both vanilloid activation of TRPV1-mediated currents and RTX binding to membranes containing native or recombinant TRPV1. This compound can also block the activation of TRPV1 by protons, anandamide, or heat. However, there are significant species differences in the potency of capsazepine at blocking TRPV1 responses evoked by nonvanilloid stimuli. Furthermore, capsazepine is known to act on other targets, including nicotinic acetylcholine receptors at micromolar concentrations. Capsazepine was reported to inhibit some pain-related behaviors in animal models, although no clinical trials have been carried out partly because of its nonspecificity. Ruthenium red is a highly charged organic cation that acts as a noncompetitive TRPV1 antagonist, apparently by blocking the channel pore. This compound, however, is even more promiscuous than capsazepine, as it blocks a number of nonselective cation channels.

Halogenated vanilloids such as iodinated resiniferatoxin (I-RTX) and N-(3-methoxyphenyl)-4-chlorocinnamide (SB36679) seem to exhibit somewhat better selectivity [41, 42]. However, they have not been employed with much success *in vivo*. One of the surprising findings arising from the *in vivo* studies of the TRPV1 antagonists is that some of the antagonists, such as N-(4-tertiarybutylphenyl)-4-(3-chlorophenyl)-1,2,3,4-tetrahydropyrazine-1(2H)-carboxamide (BCTC) and capsazepine, were also effective for mechanical hyperalgesia in a neuropathic pain model [43, 44]. These findings apparently contradict the previous results that normal mechanical nociception was observed in mice lacking TRPV1 and that TRPV1 in a heterologous expression system did not show mechanical sensitivity [45]. The possibility that TRPV1 is involved in mechanosensation in nociceptors, especially in pathological conditions, cannot be excluded. Indeed, mechanical responsiveness in the urinary bladder appears to be diminished in mice lacking TRPV1, and proteinase-activated receptor 2 (PAR 2) agonist-induced mechanical allodynia was significantly reduced in TRPV1-deficient mice. Highly selective TRPV1 antagonists will hopefully be identified through high-throughput screening in the near future, and studies using the new selective TRPV1 antagonists will address the issue of mechanosensitivity of TRPV1.

21.6 SENSITIZATION OF TRPV1

Inflammatory pain is initiated by tissue damage/inflammation and is characterized by hypersensitivity both at the site of damage and in adjacent tissue. Stimuli that normally would not produce pain do so (allodynia), while previously noxious stimuli

evoke even greater pain responses (hyperalgesia). One mechanism underlying these phenomena is the sensitization of ion channels such as TRPV1. Sensitization is triggered by extracellular inflammatory mediators that are released *in vivo* from surrounding damaged or inflamed tissues and from nociceptive neurons themselves (i.e., neurogenic inflammation) [46, 47]. Mediators known to cause sensitization include prostaglandins, adenosine, serotonin, bradykinin, and adenosine triphosphate (ATP) [48]. Tissue acidification is also induced in the context of inflammation as described above. Among the inflammatory mediators, extracellular ATP, bradykinin, prostaglandin E₂, prostaglandin I₂, trypsin, and tryptase have been reported to potentiate TRPV1 responses through metabotropic ATP receptor (P2Y)₂, B2, EP₁, IP, and proteinase-activated receptor 2 (PAR2) receptors, respectively, in a protein kinase C (PKC)-dependent manner in both heterologous expression systems and native DRG neurons [49–53]. In addition to potentiating capsaicin- or proton-evoked currents, those mediators also lower the temperature threshold for heat activation of TRPV1 to as low as 30 °C (Fig. 21.3), such that normally nonpainful thermal stimuli (i.e., normal body temperature) are capable of activating TRPV1, thereby leading to the sensation of pain. Under these circumstances, they thus can be viewed as direct activators of TRPV1. This represents a novel mechanism through which the large amounts of mediators released from different cells in inflammation might trigger a sensation of pain. The inflammatory mediator-induced TRPV1-mediated hypersensitivity has been confirmed at the whole-animal level using TRPV1-deficient mice or mice lacking the receptors of the mediators (Fig. 21.3). PKC-dependent phosphorylation of TRPV1 has been reported to be involved in the sensitization of TRPV1 by the mediators, based on the observation that several different PKC inhibitors blocked the inflammatory mediator-induced potentiation or sensitization of TRPV1 activity. Indeed, two serine residues in the cytoplasmic domain of TRPV1 were identified as substrates for PKC-dependent phosphorylation [54, 55]. There has been extensive work demonstrating the activation of a protein kinase A (PKA)-dependent pathway that influences capsaicin- or heat-mediated actions in rat sensory neurons [56–60] as well as interactions between cloned TRPV1 and PKA [61–64]. These results suggest that PKA plays a pivotal role in the development of hyperalgesia and inflammation and that a PKA-dependent pathway is also involved in TRPV1 sensitization, and PKA-dependent phosphorylation of serine residues on TRPV1, S144, S370, and S502 has been reported [64]. Further, both PKA- and PKC-dependent pathways have been reported to function on some ligands, such as serotonin and prostaglandins [50, 65]. The physiological relevance of the two different pathways downstream of serotonin or prostaglandin exposure remains to be elucidated. The fact that only PKC activation leads to the reduction of temperature threshold for TRPV1 activation might be pertinent to this issue [50]. Disruption of interaction between phosphatidylinositol-4,5-bisphosphate (PIP₂) and TRPV1 has also been reported to be involved in the sensitization of TRPV1 downstream of phospholipase C (PLC) activation by, for example, bradykinin or NGF since the amount of PIP₂, a tonic inhibitor of TRPV1, is reduced in its hydrolysis to inositol 1,4,5-trisphosphate (IP₃) and diacyl glycerol (DAG) [66, 67]. It is also known that phospholipase A₂ (PLA₂) is activated downstream of PLC activation, leading to the generation of lipoxygenase products such as 12-HPETE from AA [38]. These facts indicate three different pathways can work to modulate TRPV1 function downstream of PLC activation: a PKC-dependent pathway, a

PIP₂-mediated pathway, and a lipoxygenase product-mediated pathway. It is not currently clear which pathway is predominantly functioning in vivo.

In addition to the direct activation of TRPV1, acidification induced in inflammation also shifts the temperature response curve of TRPV1 to the left so that the channel can be activated at lower temperatures (lower than body temperature) and responses to heat are bigger at a given suprathreshold temperature. This phenomenon might also contribute to inflammatory pain. Calmodulin kinase II (CaMKII) was also reported to control TRPV1 activity upon phosphorylation of TRPV1 at S502 and T704 by regulating capsaicin binding [68]. Thus, phosphorylation of TRPV1 by several different kinases seems to control TRPV1 activity through the dynamic balance between phosphorylation and dephosphorylation. Furthermore, NGF activates the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated protein kinase (ERK), and then PI3K and ERK have been reported to sensitize TRPV1 [69]. Mechanisms underlying TRPV1 sensitization seem to be very complicated.

21.7 DESENSITIZATION OF TRPV1

Capsaicin not only causes pain but also seems to exhibit analgesic properties, particularly when used to treat pain associated with diabetic neuropathies or rheumatoid arthritis [7]. This paradoxical effect may relate to the ability of capsaicin to desensitize nociceptive terminals to capsaicin as well as to other noxious stimuli following prolonged exposure. At the molecular level, an extracellular Ca²⁺-dependent reduction of TRPV1 responsiveness upon continuous vanilloid exposure (electrophysiological desensitization) may partially underlie this phenomenon [7, 8], although physical damage to the nerve terminal and depletion of SP and CGRP probably contribute to this effect as well. Ca²⁺- and voltage-dependent desensitization of capsaicin-activated currents has also been observed in rat DRG neurons [70–73]. This inactivation of nociceptive neurons by capsaicin has generated extensive research on the possible therapeutic effectiveness of capsaicin as a clinical analgesic tool [74, 75].

Desensitization to capsaicin is a complex process with varying kinetic components: a fast component that appears to depend on Ca²⁺ influx through TRPV1 [70–73] and a slow component that does not. Calcineurin inhibitors reduce TRPV1 desensitization (the slow component), indicating the involvement of the Ca²⁺-dependent phosphorylation/dephosphorylation process [73]. In agreement with this finding, phosphorylation of TRPV1 by CaMKII was reported to prevent its desensitization [68]. In addition, PKA-dependent phosphorylation of TRPV1 has been reported to mediate the slow component of TRPV1 desensitization [61]. TRPV1 becomes dephosphorylated upon exposure to capsaicin and this phosphorylation can be restored by 8bromo-cyclic adenosine monophosphate (cAMP).

CaM has also been reported to be involved in Ca²⁺-dependent desensitization of TRPV1. CaM was found to bind to the carboxyl terminus of TRPV1. Disruption of the CaM binding segment prevented extracellular Ca²⁺-dependent TRPV1 desensitization to brief capsaicin application, although some desensitization was still observed upon more prolonged capsaicin application in cells expressing the mutant [76]. It has also been reported that CaM binds to the first ankyrin repeat in the amino terminus of TRPV1 and to be involved in desensitization [77]. Whether the amino or carboxyl terminus is more predominantly involved in Ca²⁺-dependent desensitization

by CaM is not known. Ca^{2+} -dependent desensitization is a relatively common feature of many cation channels, including cyclic nucleotide-gated channels [78], L-type Ca^{2+} channels [79, 80], P/Q-type Ca^{2+} channels [81], N-methyl-D-aspartate (NMDA) receptor channels [82, 83], and TRP channels [84, 85]. It may represent a physiological safety mechanism against a harmful Ca^{2+} overload in the cell, especially during large Ca^{2+} influx through the channels.

21.8 KNOCKOUT MOUSE STUDY

Electrophysiological analysis in a heterologous expression system revealed the importance of TRPV1 in detecting noxious thermal and chemical stimuli. To determine whether TRPV1 really contributes to the detection of these noxious stimuli in vivo, mice lacking this protein were generated and analyzed for nociceptive function [45, 86]. Sensory neurons from mice lacking TRPV1 were deficient in their responses to each of the reported noxious stimuli: capsaicin, proton, and heat. Consistent with this observation, behavioral responses to capsaicin were absent and responses to acute thermal stimuli were diminished in these mice. Pungency-related behaviors were not observed in the TRPV1-deficient mice when capsaicin was applied to their oral cavity, suggesting the involvement of TRPV1 in detection of pungency. In contrast, TRPV1 knockout mice showed normal physiological and behavioral responses to noxious mechanical stimuli, implying the existence of other mechanisms for the detection of such stimuli. The most prominent feature of the knockout mouse thermosensory phenotype was a virtual absence of thermal hypersensitivity in the setting of inflammation. These findings indicate that TRPV1 is essential for selective modalities of pain sensation and for tissue injury-induced thermal hyperalgesia.

The extent to which TRPV1 underlies the responses to noxious thermal stimuli and the contribution of other heat-sensitive channels remain to be clarified. There was a drastic reduction of heat sensitivity in DRG neurons cultured from TRPV1-deficient mice, but a small yet significant percentage of DRG neurons showed large heat-evoked current responses to heat stimuli over 55 °C. Furthermore, the TRPV1-deficient mice showed impaired responses to noxious thermal stimuli only over 50 °C, and a small but significant amount of heat-evoked *c-fos* induction persisted in spinal cord laminae I and II of TRPV1-deficient mice. These data supported the idea that other heat-sensitive channels contribute to the transmission and perception of high-intensity noxious thermal stimuli.

Recent studies have provided further support for TRPV1 involvement in inflammatory pain and have, in addition, demonstrated that the participation of TRPV1 in pain sensation may also extend to neuropathic pain, mechanical allodynia, and mechanical hyperalgesia. These conclusions are based on enhanced expression of TRPV1 in sensory neurons in the context of these conditions as well as behavioral effects of TRPV1 antagonism with capsazepine [22, 43, 49, 87, 88]

21.9 OTHER TRPV CHANNELS INVOLVED IN NOCICEPTION

TRPV2 (VRL-1) might be a potential candidate for the receptor detecting the high heat stimulus responsible for the residual high temperature-evoked nociceptive

responses observed in TRPV1-deficient mice. TRPV2 with about 50% identity to TRPV1 was found to be activated by high temperatures with a threshold of $\sim 52^{\circ}\text{C}$ [89]. TRPV2 currents showed similar properties to those of TRPV1 such as an outwardly rectifying I - V relationship, inhibition by ruthenium red, and relatively high Ca^{2+} permeability. Intense TRPV2 immunoreactivity was observed in medium- to large-diameter cells in rat DRG neurons [89–92]. In addition, many of the TRPV2-positive neurons costained with the anti-neurofilament antibody N52, a marker for myelinated neurons. Temperatures activating TRPV2 are more harmful to our body than those activating TRPV1. Therefore, expression of TRPV2 in the myelinated sensory fibers seems reasonable because A δ fibers can transmit the nociceptive information much faster than C fibers. A δ mechano- and heat-sensitive (AMH) neurons in monkey are medium- to large-diameter, lightly myelinated neurons that fall into two groups: type I AMHs have a heat threshold of $\sim 53^{\circ}\text{C}$, and type II AMHs are activated at 43°C [93]. TRPV2 expression might account for the high thermal threshold ascribed to type I AMH nociceptors. However, it has very recently been reported that nociceptors lacking TRPV1 and TRPV2 still have normal heat responses [94]. This result suggests the existence of another heat-sensitive mechanism.

Two TRPV channels, TRPV3 and TRPV4, have been found to be activated by warm temperatures, ~ 34 – 38°C for TRPV3 and ~ 27 – 35°C for TRPV4, in heterologous expression systems and to be expressed in multiple tissues, including, among others, sensory and hypothalamic neurons and keratinocytes [95–99]. TRPV4 was originally reported as an osmotically activated channel (VROAC or OTRPC4 from vanilloid receptor-related osmotically activated channel or OSM-9-like TRP channel 4, respectively) [100, 101]. Several approaches, including the knockdown of TRPV4 with gene disruption or antisense oligonucleotides, have led to reports that this protein is involved in mechanical stimulus- and hypotonicity-induced nociception in rodents [102–104]. It remains unclear whether TRPV3 is involved in nociception. However, the data that TRPV1 and TRPV3 were predicted to reside on the same chromosome and that TRPV3 was sensitized upon repeated noxious heat stimuli suggest the involvement of TRPV3 in nociception.

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OPIOID RECEPTORS

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22.1	Introduction	745
22.2	Endogenous Opioids	746
22.2.1	Enkephalins	746
22.2.2	Dynorphins	747
22.2.3	β -Endorphin	747
22.2.4	Orphanin FQ/Nociception	748
22.3	Opioid Receptors	748
22.3.1	μ Receptors	748
22.3.2	δ Receptors	751
22.3.3	κ Receptors	751
22.4	Opioid Receptor Dimerization	752
22.5	Orphanin FQ/Nociceptin and its Receptor	752
22.6	Conclusions	752
	References	753

22.1 INTRODUCTION

The use of opium, a product of the poppy plant, goes back thousands of years. The major analgesic components of opium are morphine and codeine, which are present in relatively high concentrations. Thebaine is another important compound present in opium. Although it is not active, it is widely used to synthesize a number of clinically important opioid analgesics. Following the isolation and purification of morphine and codeine, medicinal chemists synthesized thousands of analogs in an effort to avoid the side effects, which include sedation, respiratory depression, constipation, and abuse potential. Although they were not able to dissociate these actions, they did establish rigid structure–activity relationships for these drugs which, in turn, led to the proposal of distinct binding sites, or receptors, years before they were demonstrated experimentally in 1973 [1–3]. These binding sites were restricted

to nervous tissue and were highly selective for morphine and morphine-like agents. Since then, we have extended our understanding of these receptors and their endogenous ligands and have identified three classes of opioid receptors [4].

22.2 ENDOGENOUS OPIOIDS

The demonstration of opioid receptors quickly led a number of investigators to look for their endogenous ligands, eventually leading to the isolation and identification of a series of opioid peptides (Table 22.1) [5–10]. They fall into three families based upon their precursor peptides and the selectivity for the members of the opioid receptor classes. Each opioid peptide family is generated from a distinct precursor peptide encoded by three different genes. Yet, all the active opioid peptides contain the sequence Tyr–Gly–Gly–Phe–Leu or Tyr–Gly–Gly–Phe–Met as their first five amino acids. The only exception are the endomorphins (Table 22.1). These peptides, which have been isolated from brain [11], are highly μ selective. However, it is not clear whether they are synthesized de novo or generated from a precursor.

22.2.1 Enkephalins

The enkephalins were the first opioid peptides identified, and they played a major role in establishing the explosion within the field of neuropeptides. The enkephalins consist of a pair of pentapeptides that share the same first four amino acids, differing at the fifth with either a methionine (Met⁵-enkephalin) or a leucine (Leu⁵-enkephalin). They have distinct regional distributions within the brain and are the endogenous ligands for the δ receptors (see below).

Studies of the enkephalins were initially hindered by their lability, due in large part by the actions of peptidases. The demonstration that they could be stabilized by substituting a D-amino acid at position 2 led to the generation of a host of stabilized

TABLE 22.1 Opioid and Related Peptides^a

[Leu ⁵]enkephalin	Tyr–Gly–Gly–Phe–Leu
[Met ⁵]enkephalin	Tyr–Gly–Gly–Phe–Met
Dynorphin A	Tyr–Gly–Gly–Phe–Leu –Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln
Dynorphin B	Tyr–Gly–Gly–Phe–Leu –Arg–Arg–Gln–Phe–Lys–Val–Val–Thr
α -Neoendorphin	Tyr–Gly–Gly–Phe–Leu –Arg–Lys–Tyr–Pro–Lys
β -Neoendorphin	Tyr–Gly–Gly–Phe–Leu –Arg–Lys–Tyr–Pro
β -Endorphin	Tyr–Gly–Gly–Phe–Met –Thr–Ser–Glu–Lys–Ser–Gln–Thr–Pro–Leu–Val–Thr–Leu–Phe–Lys–Asn–Ala–Ile–Ile–Lys–Asn–Ala–Tyr–Lys–Lys–Gly–Glu
Endomorphin-1	Tyr–Pro–Trp–Phe–NH ₂
Endomorphin-2	Tyr–Pro–Phe–Phe–NH ₂
Orphanin FQ/ nociceptin	Phe–Gly–Gly–Phe –Thr–Gly–Ala–Arg–Lys–Ser–Ala–Arg–Lys–Leu–Ala–Asp–Glu

^aCommon sequences are highlighted.

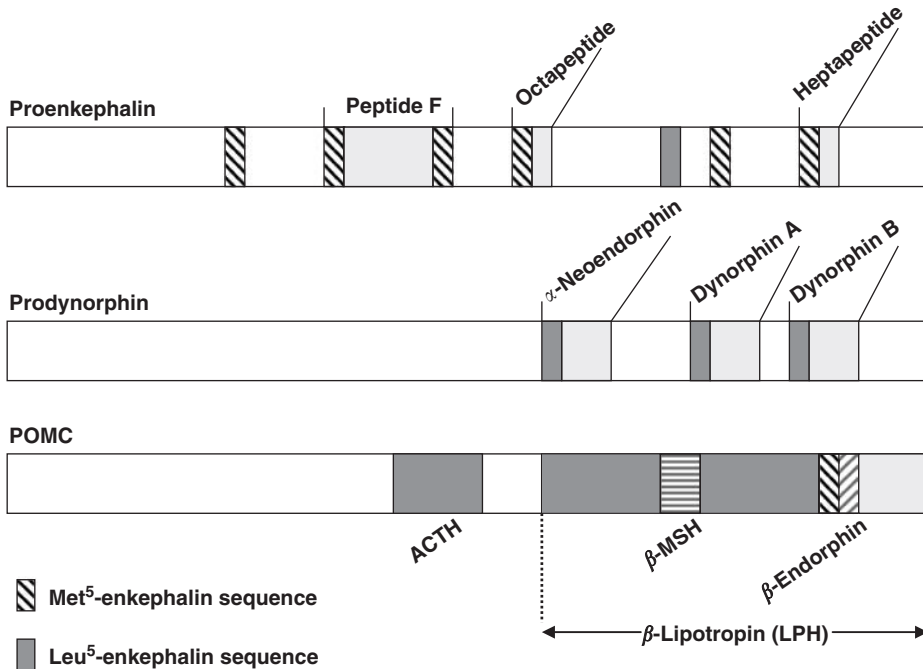


Figure 22.1 Schematic of opioid peptide precursors.

derivatives. Using these compounds, investigators have established that the enkephalins can produce analgesia through receptor mechanisms distinct from those of morphine.

The enkephalins are generated from a larger precursor that contains a number of copies of the enkephalins (Fig. 22.1). There are six copies of Met⁵-enkephalin and one copy of Leu⁵-enkephalin. However, several of the Met⁵-enkephalin may actually be contained in larger peptides (peptide F, the octapeptide, and the heptapeptide). It is not known if these extended peptides are active or simply are broken down to Met⁵ enkephalin. Thus, their significance remains unknown.

22.2.2 Dynorphins

There are three major peptides within this family: α -neoendorphin, dynorphin A, and dynorphin B. Each contains the sequence of Leu⁵-enkephalin as the first five amino acids. Dynorphin A is the endogenous ligand for κ receptors, specifically κ_1 . Although dynorphin B and α -neoendorphin also label κ receptors, there has been the suggestion that they may have a slightly different binding selectivity that may be due to the existence of κ receptor subtypes [12]. All three peptides also elicit analgesia through unique receptor mechanisms.

22.2.3 β -Endorphin

β -Endorphin has 31 amino acids, making it the largest of the opioid peptides. Yet, its N-terminus still contains the Met enkephalin sequence. As with dynorphin, there

originally were questions as to whether it was pharmacologically relevant or simply the precursor of Met⁵-enkephalin. That question was answered with the identification of the enkephalin precursor as well as that for β -endorphin, pro-opiomelanocortin (POMC), which also generates the important peptides adrenocorticotrophic hormone (ACTH) and melanocyte-stimulating hormones α -MSH and β -MSH. Unlike the other opioid peptides that are widely distributed within the central nervous system, POMC is synthesized within the brain only in the arcuate nucleus, further distinguishing it from the enkephalins. However, the major location of POMC and its peptides is within the pituitary, where β -endorphin has been shown to be coreleased with ACTH, an important stress hormone responsible for the release of cortisol. This association has suggested the possibility that β -endorphin may be involved with stress analgesia.

22.2.4 Orphanin FQ/Nociception

Although not formally a member of the opioid peptide family, orphanin FQ/nociception (OFQ/N) is strikingly similar in structure to dynorphin A. Like dynorphin A, OFQ/N contains 17 amino acids, but it contains phenylalanine at position 1 instead of tyrosine. OFQ/N labels the opioid receptor-like 1 (ORL1) receptor with very high affinity but does not compete opioid receptor binding. However, if ORL1 receptors are coexpressed with an opioid receptor, OFQ/N acquires the ability to compete opioid binding, presumably due to dimerization of the two receptor classes. Thus, it appears to be closely related to opioids, if not formally one.

22.3 OPIOID RECEPTORS

Opioid receptors were initially proposed based upon the structure–activity relationships of literally hundreds of morphine derivatives. Indeed, the concept of opiate receptors was well entrenched prior to their actual identification in binding assays in 1973 [1–4]. Three major families of opioid receptors were proposed from pharmacological studies and then confirmed in binding assays and then through cloning. Studies in the dog led Martin to propose μ (morphine-preferring) and κ (ketocyclazocine-preferring) receptors, which display high affinity for the dynorphins [13]. This was followed by the identification of δ receptors selective for the enkephalins [5, 14].

22.3.1 μ Receptors

Most of the analgesics that we use to treat pain are morphine-like and act, at least in part, through μ receptors. The μ receptors have a variety of actions. Of these, analgesia is the most commonly desired. Opioid analgesia is particularly intriguing since it does not interfere with primary sensations. Patients retain the ability to feel light touch, temperature, sharp/dull, and vibration, unlike therapy with local anesthetics. Instead, the opiates diminish the subjective “hurt” associated with pain, making these agents quite unique and valuable.

Continued use of morphine-like drugs leads to tolerance, which is defined as a diminished response with a continued dose of a drug or the need to increase the drug dose over time to maintain a response. With the opioids, this is easily seen for all

opioid actions, including analgesia, although the rate to which tolerance develops for each one action may vary. Dependence is also associated with μ -opioid use. Dependence is seen through the expression of withdrawal upon the abrupt discontinuation of the opioid or the administration of an opioid antagonist to a subject who has been maintained on an opioid for an extended period of time. The length of time and the dose of the drug determine the severity of the withdrawal syndrome, which in turn is an indication of the degree of dependence. It is important to distinguish between dependence and addiction. Whereas dependence is a physiological response seen in every subject receiving the drugs, addiction is a behavioral drug-seeking response which is actually quite rare when these drugs are used for legitimate medical purposes.

The μ opioids induce a variety of other actions, including sedation, respiratory depression, and inhibition of gastrointestinal transit. Although they have been used to treat diarrhea, this action is more commonly a problematic side-effect manifested as constipation, particularly in pain patients. Sedation is another sideeffect that can be limiting in the use of these agents. Indeed, morphine was named after the Morpheus, who was the god of sleep.

The μ -opiate receptor was first demonstrated biochemically in 1973 with the demonstration of highly selective opioid binding to membranes from the brain [1–3]. Since then, the receptor has been cloned [15–18] and has been identified as a member of the G-protein-coupled receptor family [19]. Like other G-protein-coupled receptors, μ -opioid receptors have seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus (Fig 22.2a). Following the binding of an opioid to the receptor, the receptor activates a G-protein which then induces a signaling cascade. Opioid receptors are typically inhibitory and act predominantly through G_i and G_o classes of G-proteins.

The concept of multiple μ -opiate receptors goes back 25 years [20] and is particularly important in understanding the clinical actions of these drugs [21, 22]. From the clinical perspective, multiple μ receptors help explain opioid rotation and the variability in responses, observations difficult to reconcile with a single μ -opioid receptor. In opioid rotation, patients highly tolerant to one μ opiate can be switched to a different μ opiate with a markedly enhanced analgesic response to the second drug [23]. This is due to incomplete cross tolerance, consistent with slight differences in the receptor mechanism actions of the two agents. In terms of the sensitivity, some patients respond far better to one μ opioid than another. Both observations can be replicated in animal models. In CXBK mice, morphine is a very weak analgesic with a potency 5- to 10-fold less sensitive than the traditional CD-1 animal. On the other hand, other μ opioids, such as methadone, fentanyl, and heroin, retain full analgesic activity in the CXBK animal, implying that their actions are distinct from those of morphine [4, 22].

The suggestion of multiple μ -opioid receptors has now been confirmed at the molecular level through the identification of a number of splice variants of the μ -opioid receptor MOR-1 in mice, rats, and humans [24–30] (Fig. 22.2b). MOR-1, the μ -opiate receptor that was cloned in 1993, has four exons. Exons 1, 2, and 3 encode the seven transmembrane domains of the receptor while exon 4 encodes the last 12 amino acids in the C-terminus. The major series of splice variants in all three species involve variants that are alternatively spliced downstream of exon 3, with a variety of different exons in place of exon 4. The differences among these variants is restricted to the tip of the C-terminus (Fig. 22.2c). Although some variants share

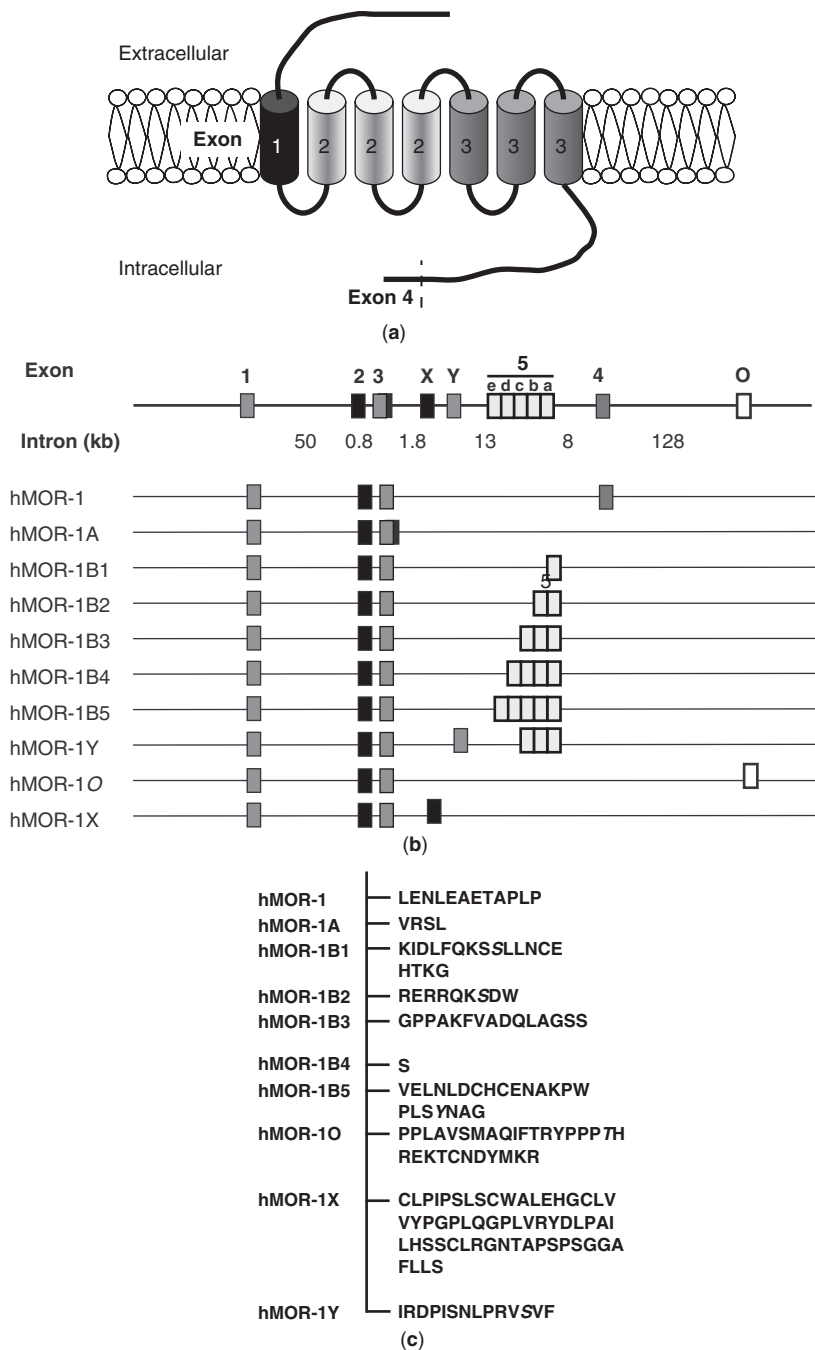


Figure 22.2 Schematic of MOR-1 (a) MOR-1 within membrane. The exons responsible for coding the different regions are indicated. (b) Alternative splicing of MOR-1 in humans. Note that only full-length variants are shown, which involve splicing at the 3' end. There is another series of 5' spliced variants. (c) Amino acid sequences of alternatively spliced C-terminus of human MOR-1 variants downstream from exon 3.

these spliced exons, all the amino acid sequences downstream of exon 3 are unique and many are identical or highly homologous among species. The binding pockets responsible for the docking of the opioid ligands within the receptor are defined by the seven transmembrane domains. Thus, all these C-terminus splice variants have identical binding pockets, explaining their similar affinities for μ opioids and their high selectivity for μ drugs. However, these changes in the C-terminus do influence the transduction systems and impact on both the efficacy and potency of drugs acting through them [24–26, 29, 31]. In mice, these C-terminus variants also have different distributions regionally and even within cells [32–35]. Furthermore, one variant, MOR-1C, is associated with calcitonin gene-related peptide (CGRP) while MOR-1 is not [32].

In mice, and possibly humans and rats, there is a second series of splice variants at the N-terminus that generate truncated proteins that lack all seven transmembrane domains seen in MOR-1 [28]. The potential pharmacological significance of these variants is not clear, but they have been demonstrated in the brain at both the messenger RNA (mRNA) and protein levels and there are indications that they may be pharmacologically irrelevant.

22.3.2 δ Receptors

The δ receptors were first proposed from studies examining the pharmacology of the enkephalins [14], which are their endogenous ligand. Since then a wide range of synthetic peptides and nonpeptides that are selective for δ receptors have been developed and widely used to characterize the actions of these receptors in animal models. However, there are not any clinically available δ ligands at present and our understanding of their actions in humans is not yet well understood.

The δ receptors are members of the G-protein-coupled receptor family and show marked homology to the μ receptor, particularly in the transmembrane domains. However, the δ receptors are unique in terms of their binding selectivity and are readily distinguished from μ receptors. They are expressed in different regions of the brain and are presumably involved in a variety of distinct physiological responses.

The δ -opioid receptor DOR-1 also has seven transmembrane domains encoded by three exons [36, 37]. Unlike MOR-1, the C-terminus of DOR-1 ends within the third coding exon and there is no C-terminus splicing. Several splice variants of DOR-1 have been reported, but they do not appear to be pharmacologically relevant [38, 39].

22.3.3 κ Receptors

The κ receptors were first proposed from the benzomorphan ketocyclazocine, but we now know that their endogenous ligand is dynorphin A. The κ ligands have been examined clinically and several are available, including pentazocine, nalorphine, and nalbuphine. These early κ drugs were mixed κ agonists– μ antagonists. Studies with more selective κ drugs have confirmed their analgesic activity, but the drugs proved problematic due to their psychotomimetic effects, actions that were also seen with the mixed agonist–antagonist drugs. The highly selective κ drugs that were in clinical trials have been withdrawn.

The κ receptors are also members of the G-protein-coupled receptor family. They were cloned soon after the δ receptors [40–42]. The cloned κ -opioid receptor KOR-1

has three coding exons that encode a seven-transmembrane receptor and the transmembrane domains have high homology with the other members of the opioid receptor family. However, its binding selectivity and distribution within the brain are distinct.

22.4 OPIOID RECEPTOR DIMERIZATION

G-protein-coupled receptors have an additional layer of complexity due to their tendency to associate with themselves (homodimers) or other G-protein-coupled receptors (heterodimers) [43, 44]. Similar observations have been made with opioid receptors [45–48]. In some cases, heterodimers take on pharmacological characteristics unlike those of either receptor expressed alone [46–48]. Thus, this ability to interact with additional receptors to generate pharmacologically unique dimers extends the number of potentially important opioid receptors manyfold. To make matters even more difficult, opioid receptors also can associate with receptors of other classes, as shown between opioid and β -adrenergic and α -adrenergic receptors [49, 50]. Thus, the presence of three opioid receptors can lead to a host of novel receptor classes due to interactions with other receptors.

22.5 ORPHANIN FQ/NOCICEPTIN AND ITS RECEPTOR

There is a fourth receptor with high homology to the traditional opioid receptors, termed ORL-1 [51–56], which is the receptor for a novel peptide termed nociceptin or orphanin FQ [57, 58]. This 17-amino-acid peptide is distinct from the opioid peptides in that its first amino acid is phenylalanine rather than tyrosine. It displays no appreciable affinity for the traditional opiate receptors. However, the high homology of the ORL-1 receptor with the opioid receptors suggests a common ancestry. Interestingly, OFQ/N is important in the modulation of pain. At low concentrations, OFQ/N is hyperalgesic and enhances pain perception, whereas higher doses are analgesic. The analgesic actions of OFQ/N are reversed by opioid antagonists, which is somewhat unexpected since OFQ/N itself does not bind to opioid receptors. However, when coexpressed, MOR-1 and ORL-1 receptors dimerize and display a unique pharmacological profile in which opioids and OFQ/N can displace each other with a high potency not seen when each receptor is displayed alone [48].

22.6 CONCLUSIONS

The opiate peptides and opiate receptors comprise a highly complex system within the central nervous system (CNS). Although intimately involved with analgesia and pain modulation, they have a host of other functions. Three classes of opioid receptors were proposed from classical pharmacological studies and each family has been cloned. Early work proposing multiple μ -opioid receptors also have been confirmed with the isolation and identification of a host of splice variants of the μ -opioid receptor MOR-1. The complexity of the opioid system is further illustrated by the tendency of opioid receptors to dimerize and associate with other opioid receptors as well as unrelated classes of G-protein-coupled receptors.

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23

ADVENT OF A NEW GENERATION OF ANTIMIGRAINE MEDICATIONS

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23.1	Overview	758
23.2	What Is Migraine?	758
23.2.1	Historical Perspective	758
23.2.2	Definition of Migraine	758
23.3	Role of CGRP in Migraine	759
23.3.1	Neurovascular Model	759
23.3.2	CGRP Synthesis and Actions	760
23.3.3	Multiple Sites of CGRP Action in Trigemino-vascularity	761
23.3.4	Injection of CGRP Induces Migrainelike Headaches	763
23.3.5	Therapeutic Efficacy of CGRP Receptor Antagonist	763
23.4	Pharmacological Management of Migraine	764
23.4.1	Acute Therapy	764
23.4.1.1	Triptans	764
23.4.1.2	Nonsteroidal Anti-Inflammatory Drugs and Nonopioid Analgesics	765
23.4.1.3	Opioids	765
23.4.1.4	Ergots	765
23.4.1.5	Valproate	765
23.4.1.6	Antiemetics	765
23.4.2	Preventive Therapy	766
23.4.2.1	Anticonvulsants	766
23.4.2.2	β -Blockers	767
23.4.2.3	Antidepressants	767
23.4.2.4	Calcium Channel Blockers	767
23.4.2.5	Nonsteroidal Anti-Inflammatory Drugs	768
23.4.2.6	Botulinum Toxin	768
23.4.2.7	Other	768
23.5	Future Directions	768
23.5.1	Potential for Targeted Repression of CGRP Synthesis	768
23.5.2	Potential for CGRP Antagonists and Migraine Drugs for Other Forms of Pain	768
	References	769

23.1 OVERVIEW

This chapter will address recent developments in the treatment of migraine. The recent promising clinical trials using a new antimigraine medication, BIBN4096BS (see Fig. 23.3 below), heralds the advent of a new generation of therapeutics. BIBN4096BS targets the receptor for the neuropeptide calcitonin gene-related peptide (CGRP), which appears to play a causal role in migraine pain. We will discuss the rationale and therapeutic implications of modulating CGRP, including its potential for acting at multiple sites in the trigeminovascular system. We will then discuss the currently used acute and preventative pharmacological treatments. Of particular interest is the increasing evidence that antiepileptic drugs may be an effective prophylactic treatment for migraine. Future strategies to selectively downregulate CGRP synthesis and the potential use of migraine drugs for other forms of trigeminal-mediated pain, such as temporomandibular disorder and trigeminal neuralgia, will be explored.

23.2 WHAT IS MIGRAINE?

Migraine is a debilitating chronic episodic disorder characterized by attacks of debilitating headaches and other associated symptoms. Migraine affects 12% of the general population at least once a year [1, 2]. It is estimated that the lifetime prevalence may be as high as 18% [3]. It is one of the most underdiagnosed and undertreated neurological diseases [4]. In this section, we will provide a brief review of the history and the now-accepted clinical diagnostic criteria of migraine.

23.2.1 Historical Perspective

People have been suffering from migraine for millennia. The first evidence of migraine is suggested by neolithic skulls from 7000 BCE that show signs of trephination, an ancient procedure that has been used to treat migraine as late as the mid-seventeenth century [5]. Descriptions of migrainelike pain have been found in Babylonian tablets dating back to 3000–4000 BC and Egyptian papyri from 1550 BC [6]. We owe to Hippocrates (c. 400 BCE) the recognition of the syndrome now known as migraine. The term *migraine* originates from the Greek word *hemicrania*, provided by Galen in the second century AD. Despite this long history, it has really only been since the 1990s that significant therapeutic inroads have been made in our understanding and treatment of migraine.

23.2.2 Definition of Migraine

The International Headache Society (IHS) developed in 1988 and reviewed in 2004 a classification system for headaches [7, 8]. But until recently these criteria have been used loosely to define migraine for research purposes. Migraines are divided in two major groups, migraines without aura and migraines with aura. Migraines without aura are defined as headaches lasting 4–72 h (untreated or unsuccessfully treated) with at least two of the following: (1) pulsating quality, (2) unilateral location, (3) moderate or severe intensity, and (4) aggravation by routine physical activity. It has been suggested by some physicians that these criteria can be remembered by the first

initial as a “puma” chewing on your brain. In addition to these core criteria, migraine also must fulfill at least one of the following two criteria: (1) nausea and/or vomiting and (2) photophobia and phonophobia. An important point for considering the mechanism behind the pain is that the untreated headache can last for days.

An aura consists in focal neurological phenomena that precede or accompany the headache. The aura can be visual, sensory, or motor and may involve language or brain stem disturbances. Migraines with aura are less common and occur in about 15% of migraineurs [3], although an estimated 30% of migraineurs experience auras at some time [9].

Migraine is a chronic condition, with episodic attacks of variable frequency, that disrupts normal daily activities and has severe economic repercussions [10–12]. Many patients rely on nonprescription medications such as aspirin, acetaminophen, and ibuprofen. However, they often are not effective when pain is moderate to severe and are commonly overused [13, 14]. This can lead to chronic headaches, or “rebound headache.” Migraines are more frequent during the most productive years of a person’s life. Based on a survey by the World Health Organization, severe migraine was rated as one of the most disabling chronic disorders [15].

There is a marked sex bias among migraineurs [1]. Migraine occurs in 18% of women, in contrast to a 6% frequency in men. In children prior to puberty, the frequency of migraine is about 6% for both boys and girls. After menopause, many, but not all, women report a decrease in migraines. One of the most common triggers of migraines is the timing during estrus cycle. While a molecular explanation for this link has not been forthcoming, several mechanisms have been proposed [16].

23.3 ROLE OF CGRP IN MIGRAINE

A causal role for CGRP during the painful phase of migraine has been a topic of speculation for the past 15 years. In this section, we will describe how CGRP fits into the currently prevailing model for migraine. We will briefly describe the synthesis and known activities of CGRP that support the hypothesis that CGRP contributes to the painful phase of migraine. We will then describe the conclusive evidence from two clinical studies showing that administration of CGRP causes a headache and that a CGRP receptor antagonist can block migraines.

23.3.1 Neurovascular Model

Migraine is now generally accepted to be a neurovascular disorder involving the meningeal and cerebral blood vessels of the cranial vasculature [17–19]. By neurovascular disorder, it is meant that dilation of blood vessels and pain are triggered by neural rather than vascular signals [20, 21]. The identity of those neural signals remains undefined. However, activation of serotonergic and noradrenergic brain stem nuclei has been detected by positron emission tomography during migraine [22, 23]. This suggests the possibility of dysfunction of the aminergic neurons of the brain stem that modulate the craniovasculature. Indeed, given the heterogeneity of migraine, it seems possible that there will be multiple mechanisms by which the pain may be triggered.

Whatever the initial trigger, it is clear that a key player in the pain pathway is the trigeminal ganglion. Nerves from the trigeminal ganglion control cerebral blood flow and provide the pain-sensitive sensory innervation of the cerebrovasculature [24–26]. The trigeminal ganglion is also the major source of CGRP innervation of craniofacial structures and the cerebrovasculature [24, 27]. The ganglion is a heterogeneous collection of neurons with CGRP in ~25% of the neurons, primarily in unmyelinated nociceptive fibers [28]. The importance of trigeminovascular CGRP is highlighted by a report that human cerebral arteries are 10 times more sensitive than coronary arteries to CGRP [29].

While the role of the trigeminal nerves in migraine is increasingly evident, the link between the initiation of migraine and the invocation of the trigeminovascular system is not known. The prevailing current theory is that migraine is initiated by a dysfunction of brain stem neurons that modulate craniovascular sensory input [17]. According to this model, this dysfunction leads to changes in meningeal blood vessel dilation and a reflexlike response of perivascular trigeminal nerves [17, 19]. A complementary model has been proposed in which a wave of cortical spreading depression leads to activation of parasympathetic and trigeminal afferents, which then causes vessel dilation and nociception [30]. Cortical spreading depression is associated with the aura that precedes some migraines [31–33]. This model does not account for migraine without aura, but the authors speculate that the aura may be subclinical. Either way, it is intriguing that a genetically engineered mouse that contains a calcium channel mutation found in familial hemiplegic migraine patients, who tend to have auras, is more susceptible to cortical spreading depression [34]. Mutations in the P/Q calcium channel and Na^+ , K^+ –adenosine triphosphatase (ATPase) have been identified in these patients [35, 36]. Whether this mouse model has elevated trigeminal activity or CGRP release remains to be seen.

The neurovascular model is consistent with clinical evidence that CGRP levels are elevated in the jugular outflow of migraineurs and decreased upon treatment with triptan antimigraine drugs, coincident with pain relief [4, 17, 37]. Activation of trigeminovascular afferents in the meninges and at the major vessels leads to release of CGRP substance P and neurokinin A. These peptides are known to cause neurogenic inflammation. Neurogenic inflammation is the “sterile” inflammatory response involving vasodilation, plasma extravasation (vessel leakage), and mast cell degranulation [38]. Neurogenic inflammation has been demonstrated upon trigeminal activation in animals, but it has not been established in migraine patients [17]. Release of CGRP and other neuropeptides at the efferent terminals in the brain stem contributes to nociception. This involves central sensitization to lower pain response thresholds and allodynia, which causes previously innocuous stimuli, such as combing of hair, to be painful [39–41]. Thus, peripheral CGRP release is believed to cause vasodilation and neurogenic inflammation and release in the brain stem helps relay nociceptive signals to the central nervous system (CNS) in migraine [42, 43].

23.3.2 CGRP Synthesis and Actions

CGRP was discovered over 20 years ago as an alternative RNA splicing product from the calcitonin gene (Fig. 23.1) [44]. CGRP is prominent in nerve fibers surrounding peripheral and cerebral blood vessels and it is the most potent peptide

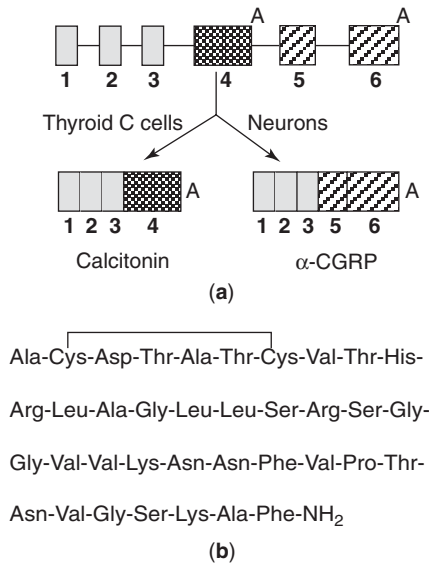


Figure 23.1 Schematic of calcitonin/α-CGRP gene. (a) Alternative processing of primary transcript in thyroid C cells yields primarily calcitonin messenger RNA (mRNA), while CGRP mRNA is the primary product in neurons. The alternative polyadenylation sites following exons 4 and 6 are indicated. (b) Primary sequence of human α-CGRP peptide. The disulfide bond is indicated.

dilator known [24, 45–47]. This vasodilatory activity has been confirmed in two of three lines of CGRP knockout mice [48, 49]. Coincident with vasodilation, CGRP plays an important role in neurogenic inflammation and nociception. CGRP triggers mast cell release of proinflammatory cytokines and inflammatory agents [47]. In a study using homozygous CGRP null mutant mice, CGRP was required for somatic and visceral pain signals associated with neurogenic inflammation [50]. In addition to its actions at the afferent terminals, CGRP modulates nociceptive input via central pathways. Notably, injection of CGRP into the trigeminal nucleus of the brain stem elicits a cardiovascular response that is similar to painful stimuli [51, 52].

Consistent with these biological activities, abnormal levels of CGRP have been implicated in hypertension, migraine, subarachnoid hemorrhage, and myocardial infarction [53]. As described below, the ability of CGRP to induce headache [54] and a CGRP receptor antagonist to alleviate migraine [55] firmly establish the importance of elevated CGRP in migraine pain [42, 43, 56]. While the objective of clinical efforts has been to reduce CGRP actions, elevated CGRP can also be beneficial. For example, following myocardial infarction it is believed that elevated CGRP release plays a protective role during ischemia [57–59].

23.3.3 Multiple Sites of CGRP Action in Trigemino-vascularity

There are three potential sites of action for CGRP (Fig. 23.2). The best characterized site of CGRP action is on the vasculature. Human cerebral vessels have functional CGRP receptors and the vascular smooth muscle cells express the calcitonin

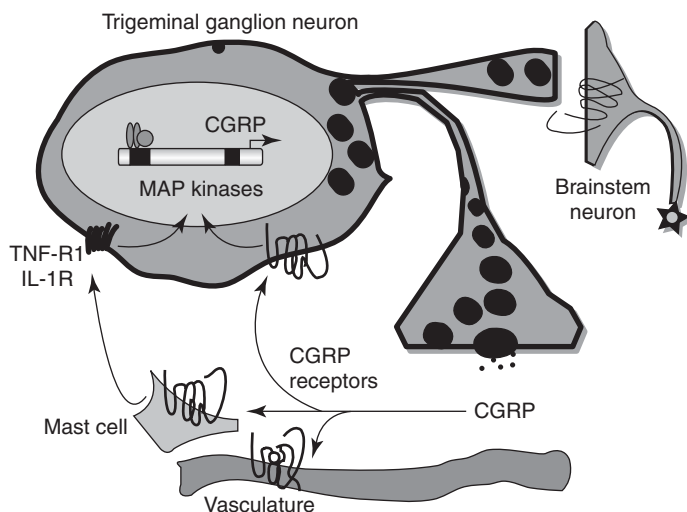


Figure 23.2 Model depicting likely sites of action of CGRP and CGRP antagonist drugs in trigeminovascularity. A pseudounipolar trigeminal ganglion neuron innervating meningeal vessels and projecting to the brain stem is depicted. (1) CGRP receptors are present on the smooth muscle and endothelium of the vasculature. (2) Receptors are present on dural mast cells that can release cytokines and inflammatory agents. (3) There are CGRP receptors on the ganglion neurons and on secondary neurons in the brain stem. The CGRP receptor is shown as a seven-pass transmembrane protein with the associated single-pass RAMP1 protein. Signals from cytokine receptors and the CGRP receptors are predicted to activate mitogen-activated protein (MAP) kinase cascades that stimulate CGRP enhancer activity. The known enhancer elements are indicated.

receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) proteins that comprise the CGRP receptor [60, 61]. Inhibition of vascular CGRP receptors by the CGRP antagonist BIBN4096BS was shown to inhibit CGRP-induced dilation of the human middle cerebral and middle meningeal arteries [62]. In addition to actions on major vessels, CGRP and presumably the antagonist can also act on receptors located on pial arterioles. Dilation of these meningeal vessels and the associated neurogenic inflammation are believed to be important events in migraine.

The second potential site of CGRP action is on receptors on dural mast cells. CGRP is known to induce mast cell degranulation. Mast cells release histamine and other inflammatory agents and proinflammatory cytokines. In this capacity, the CGRP antagonist should block both meningeal vessel dilation and neurogenic inflammation.

The third potential site is on neurons. There are CGRP receptors on the primary sensory trigeminal ganglion neurons [61] and the second-order sensory neurons within trigeminal nuclei in the caudal brain stem and upper cervical spinal cord [39, 63]. CGRP binding to the ganglion neurons might potentially create a positive-feedback loop with increased CGRP synthesis and secretion. This hypothesis is currently being tested. Inhibition of these CGRP actions by the BIBN4096BS drug would likely reduce the persistent activation of the ganglion neurons. CGRP action on brain stem neurons is known to be involved in relaying nociceptive signals to the

thalamus. BIBN4096BS would likely reduce this nociceptive signaling and the sensitization of second-order neurons that is believed to contribute to migraine pain. Inhibition of the trigeminovascular system at either or both of these sites would also be expected to diminish the allodynic effects and effects from autonomic parasympathetic ganglia that are associated with migraine [40].

23.3.4 Injection of CGRP Induces Migrainelike Headaches

The possible causative role of CGRP in migraine has been studied in a double-blind crossover study. Twelve subjects, all of them migraineurs, were administered human CGRP or placebo intravenously on two different days. Two patients developed hypotension and one had an infection, and these three subjects were excluded. Eight of nine patients had immediate headache, within 40 min of the infusion of CGRP versus only one of nine treated with placebo. This immediate headache did not fulfill criteria for migraine. All nine patients had a delayed headache, from 1 to 12 h after the beginning of the infusion of CGRP versus only one receiving placebo. In three of the nine patients treated with CGRP, the delayed headache fulfilled the criteria for migraine [54].

23.3.5 Therapeutic Efficacy of CGRP Receptor Antagonist

The final proof that CGRP plays a causal role in migraine pain is the recently reported efficacy of a novel nonpeptide CGRP receptor antagonist, BIBN4096BS (Fig. 23.3) [55]. Doods and colleagues [64] identified BIBN4096BS as a potent and highly specific antagonist to human CGRP receptors (Boehringer Ingelheim) [29, 62, 64]. BIBN4096BS binds to the human CGRP receptor with over 100-fold higher affinity than the CGRP receptor in other species [29, 64]. BIBN4096BS binds the CLR/RAMP1 protein complex, and the species selectivity appears to be due to BIBN4096BS interactions with RAMP1 [65, 66].

The antagonist BIBN4096BS was shown to be effective in the acute treatment of migraine headache in an international, multicenter, randomized, double-blind European clinical trial [55]. A total of 126 migraineurs were randomized to receive BIBN4096BS or placebo. The patients treated with BIBN4096BS had both decreased headache and improvements in the associated symptoms of nausea, photophobia, phonophobia, and inability to function. The main side effect to the drug was mild

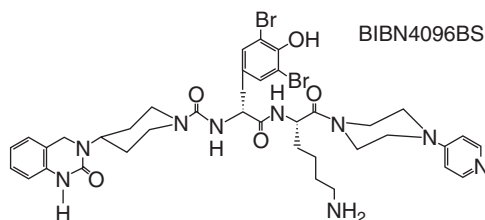


Figure 23.3 Structure of nonpeptide CGRP antagonist BIBN4096BS. BIBN4096BS, developed by Boehringer Ingelheim [64], is schematically represented (1-piperidinecarboxamide, *N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1 piperazinyl]carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-, [R-(R*,S*)]-).

paresthesias that occurred in 7% of the subjects; the other side effects, nausea, headache, dry mouth, and abnormal vision, were very infrequent, 2% each. BIBN4096BS was also effective at treating CGRP-induced headache [67]. Importantly, the drug does not appear to have any adverse cardiovascular effects [68].

The significance of this proof-of-principle result is twofold. First, it presages the development of other CGRP receptor antagonists and agents that interfere with CGRP synthesis or action. Second, the CGRP antagonist is not likely to have the cardiovascular side effects of the currently used triptans.

23.4 PHARMACOLOGICAL MANAGEMENT OF MIGRAINE

The Quality Standards Subcommittee of the American Academy of Neurology developed a practice parameter for physicians in 2000, summarizing the results from evidence-based reviews on the pharmacological, including acute and preventive, as well as nonpharmacological treatments and diagnostic evaluation for migraine patients [69]. This is a very useful resource, but a review of all the drugs would be too extensive for this chapter. We will focus in the following sections on some of the most commonly used drugs included in these guidelines.

23.4.1 Acute Therapy

When a migraine attack occurs, abortive medications are used to arrest the attack or at least decrease the severity and duration of the pain and associated symptoms. An ideal abortive drug should have a rapid onset and long-lasting effect, should be easily administered, preferably orally, and should lack side effects and interactions with other medications. One must also keep in mind the cost of abortive drugs, which ranges from \$0.01/unit (generic aspirin) to approximately \$15/unit (triptans) and can be as high as \$64/unit [dihydroergotamine-45 intra muscular (DHE-45 IM)] [70].

23.4.1.1 Triptans. Perhaps the most significant advance in migraine therapy was the antimigraine drug sumatriptan in the early 1990s. Sumatriptan and the soon-to-follow related triptan drugs provide pain relief for about 60% of migraineurs within 2 h [71]. However, only about 30% of the patients were fully pain free at 2 h and only 20% were still pain free after 24 h. Thus, there is room for improved therapies.

Sumatriptan provided important clues to the underlying mechanisms of migraine pathology [17, 20, 72]. The triptans recognize the 5-hydroxytryptamine (5-HT)_{1B}, 5-HT_{1D} and 5-HT_{1F} serotonin receptors [38, 73]. At the time of the development of sumatriptan, the 5-HT₁ receptors were only known to be on blood vessels. Since then functional 5-HT_{1B}, D, F receptors have been found on perivascular trigeminal nerve terminals and the trigeminal nucleus caudalis in the brain stem [39, 74–76]. Consistent with the multiple locations of the 5-HT₁ receptors, triptans can inhibit vasodilation of intracranial vessels and inhibit CGRP release to block neurogenic inflammation and central transmission of nociceptive stimuli from trigeminal nerves [38]. The ability of triptan drugs to lower CGRP levels coincident with pain relief is consistent with the causal role of CGRP in migraine discussed in the previous section.

Triptans are also able to repress transcription of the CGRP gene in cultured trigeminal ganglion neurons [56, 77]. Transcriptional repression was seen after 2–4 h,

which is close to the estimated half-life of sumatriptan and within the 12 h required for clearance from the body, especially with the newer triptans [17, 78]. While it is risky to extrapolate from cells to people, we speculate that transcriptional repression might lower CGRP levels over the long term. This may be relevant in triptan overuse syndromes, possibly during the withdrawal headache [79, 80].

Unfortunately, the triptans are not miracle drugs. As mentioned above, about one-third of patients do not respond to triptans. In addition, there is a high recurrence rate of the headache [81]. Most importantly, there are potentially lethal side effects. The triptans can cause severe vasoconstriction of coronary arteries [82]. Because of this, triptans are contraindicated in patients with established cardiovascular disease, and they should be used cautiously in patients with cardiovascular risk factors. It is also very important to be aware of the fact that frequent use of triptans is associated with chronic headaches, which proves to be a very disabling condition with poor response to therapy [80]. There are currently seven different triptans in the market. Listed in alphabetic order these are Almotriptan, Eletriptan, Frovatriptan, Naratriptan, Rizatriptan, Sumatriptan, and Zolmitriptan. Their relative efficacies have recently been compared in a meta-analysis of 53 trials [71].

23.4.1.2 Nonsteroidal Anti-Inflammatory Drugs and Nonopiate Analgesics. The most commonly used abortive drugs are likely to be over-the-counter analgesics, nonsteroidal anti-inflammatory drugs, and combination of analgesics with caffeine. These have proven to be effective but are not free of side effects, especially gastrointestinal symptoms. Another important aspect to consider is the risk of chronic daily headaches associated with frequent use of analgesics [83–86].

23.4.1.3 Opioids. Opioids have variable efficacy and are usually reserved as rescue medications or for specific situations like pregnancy, coronary artery disease, or other comorbidities that preclude the use of other drugs.

23.4.1.4 Ergots. Ergotamine and dihydroergotamine have serotonin agonist activity and are vasoconstrictors. Dihydroergotamine nasal spray has shown to be effective and well tolerated; other preparations are intramuscular, intravenous, oral tablet, sublingual, and suppository [87, 88].

23.4.1.5 Valproate. Intravenous valproate is also used as abortive treatment in migraines, but its effectiveness needs to be confirmed in randomized, double-blinded, placebo-controlled studies. The doses used in clinical practice are generally well tolerated [89, 90].

23.4.1.6 Antiemetics. Several antiemetic drugs are frequently used in acute migraine either to treat the associated nausea or for their effectiveness on the overall attack. The most common side effect seen with these drugs is sedation. Chlorpromazine, metoclopramide, and prochlorperazine, preferably in intravenous formulation, can be used, although there are some contradictory data [69, 94]. Antiemetics can also increase the effectiveness of other oral abortive drugs by improving delayed gastric emptying associated with acute attacks of migraine [95].

23.4.2 Preventive Therapy

Preventive medications are drugs taken on a daily basis to decrease the frequency, severity, and duration of attacks as well as to improve their responsiveness to abortive treatment and reduce disability. Many migraineurs require a prophylactic approach due to frequent or very debilitating attacks, lack of response to abortive treatment, or inability to use acute drugs due to comorbidities or interaction with other medications. Several groups of medications are used for preventive treatment of migraines: anticonvulsants, antidepressants, β -adrenergic blockers, Ca^{2+} channel blockers, botulinum toxin, and others.

23.4.2.1 Anticonvulsants. Migraines commonly coexist with other chronic neurological and psychiatric conditions. Some of these disorders, such as epilepsy, are also characterized by episodic attacks like migraines. Individuals with epilepsy have a twofold higher risk of suffering from migraine than those without epilepsy. Migraineurs have a higher risk of epilepsy than nonmigraineurs [96].

Several anticonvulsants drugs are also used for migraine prophylaxis. Only two are approved by the Food and Drug Administration (FDA) for such indication. Antiepileptic drugs are used for trigeminal neuralgia and are increasingly being used in migraine therapy [97–100]. While their mechanisms vary, many act via the γ -aminobutyric acid (GABA) system. For example, valproate elevates GABA levels [100]. Indeed, some trigeminal ganglion neurons contain GABA and many have GABA_A receptors [100]. We have found that both GABA_A and GABA_B receptors are present on cultured trigeminal ganglion neurons (unpublished observations). Whether any of these drugs act directly on the trigeminal nerves is speculative. It is generally believed that GABAergic antiepileptics act by inhibiting neural activity in the CNS. The study of these antiepileptic drugs is especially interesting because a better understanding of their mechanisms of action could throw some light on the pathophysiological mechanisms underlying migraine.

Valproate was the first antiepileptic drug approved by the FDA for migraine prophylaxis [101, 102]. The most common side effects of valproate are gastrointestinal, although asthenia, weight gain, alopecia, and tremor are also relatively frequent. Rarely, more severe idiosyncratic adverse reactions like hepatitis, pancreatitis, or hematological disorders can occur. Valproate is potentially teratogenic. An extended-release preparation allows taking valproate only once a day. The starting dose for valproate and all the anticonvulsants is followed by slow titration to an increased dosage.

Topiramate has shown efficacy in migraine prevention and has been the second and most recent antiepileptic drug approved by the FDA for this purpose [103–106]. Although the exact mechanism of action of topiramate is not fully understood, it is probably related to modulation of GABA and glutamate-mediated neurotransmission as well as its direct activity on α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate glutamate receptors [97, 98]. The most common side effects are paresthesias, cognitive symptoms and sedation, nausea, anorexia, and weight loss.

Gabapentin is used for several neuropathic conditions as well as for migraine prophylaxis, although more studies are needed to support its use in migraine [69, 107]. Similar to other antiepileptics, Gabapentin is likely to have multiple mechanisms of

action, but these are not completely understood [97]. The most common side effects are somnolence and dizziness, which may be minimized by slow titration.

23.4.2.2 β -Blockers. The nonselective β blockers propranolol, timolol, and nadolol as well as the selective β_1 blockers atenolol and metoprolol are used in prevention of migraine. Their mechanism of action, although remaining unknown, is thought to be central, at least in part due to β_1 adrenoreceptor antagonism in the ventroposteromedial thalamus [108, 109].

The use of propranolol or timolol appears to be more effective than the other β blockers [110, 111]. Fatigue, depression, nausea, dizziness, and insomnia are the most frequent side effects, although they are usually well tolerated. Nonselective β blockers should not be used in patients with asthma or diabetes. A long-acting formulation of propranolol facilitates compliance by allowing once-a-day administration [112].

23.4.2.3 Antidepressants. While the mechanism of antidepressant drugs is not well understood, it is not thought to be due to its effect on underlying depression [113], although more studies are needed to confirm this [114]. Antidepressants produce a decrease in β -adrenergic receptor density and norepinephrine-stimulated cyclic adenosine monophosphate (cAMP) response [87].

Several tricyclic antidepressants (TCAs) are used for migraine prophylaxis: amitriptyline, nortriptyline, protriptyline, doxepin, and imipramine. TCAs upregulate the GABA_B receptor, downregulate the histamine receptor, and enhance the neuronal sensitivity to substance P. Some TCAs are 5-HT₂ receptor antagonists. Absorption, distribution, and excretion of TCAs are very variable; thus the dose must be individualized. These drugs frequently cause side effects due to their antimuscarinic, antihistaminic, and α -adrenergic activity. They should be used with caution in elderly patients and avoided in patients with arrhythmias due to potential cardiac toxicity. Amitriptyline is the best studied TCA and its use is recommended based on multiple consistent randomized clinical trials [69]. Nortriptyline is a metabolite of amitriptyline and is less sedating.

A second group of antidepressants used in migraine prophylaxis are the selective serotonin reuptake inhibitors (SSRIs): fluoxetine, fluvoxamine, paroxetine, sertraline, and citalopram. All of them have been used with variable results in prevention of migraine. These drugs have less antimuscarinic, antihistaminic, and cardiovascular side effects than TCAs, but the quality of evidence to support their recommendation is not as high as for TCAs [69].

Monoamine oxidase inhibitors (MAOIs) are a third group of antidepressants used in prevention of migraines. Phenelzine, a nonspecific inhibitor of MAO_A and MAO_B, is considered effective in migraine prophylaxis by experts, but the scientific data available are insufficient. Common side effects of MAOIs are insomnia, orthostatic hypotension, constipation, weight gain, and peripheral edema. Patients on MAOIs must follow a tyramine-restricted diet and avoid certain medications to prevent hypertensive crisis.

23.4.2.4 Calcium Channel Blockers. The strength of evidence to support the use of verapamil and nimodipine for prevention of migraines is not very strong, but these are relatively commonly used drugs. Similarly to what occurs with other prophylactic medications, the physiological mechanisms of action of calcium channel antagonists

are unclear. Verapamil is usually well tolerated. Constipation is one of the most common side effects. A sustained-release preparation facilitates compliance.

23.4.2.5 *Nonsteroidal Anti-Inflammatory Drugs.* Aspirin, ketoprofen, and naproxen can be used in prevention of migraine, although they are not as effective as some of the drugs previously mentioned and they might have significant gastrointestinal and renal side effects [108].

23.4.2.6 *Botulinum Toxin.* Pericranial injections of botulinum toxin type A (Botox) have recently been introduced as an alternative preventive option for migraine prophylaxis. The evidence available is somewhat inconsistent and further well-designed clinical trials are needed [91, 115].

23.4.2.7 *Other.* Feverfew is a medicinal herb with undetermined clinical effectiveness. Magnesium supplementation has been studied with contradictory results. The most common side effects were diarrhea and gastric irritation [116]. Riboflavin (vitamin B₂) at high doses is well tolerated and can be used for migraine prevention [117].

23.5 FUTURE DIRECTIONS

23.5.1 Potential for Targeted Repression of CGRP Synthesis

An understanding of the mechanisms by which trigeminal CGRP levels are regulated will allow the development of strategies to specifically lower CGRP levels for the treatment of craniofacial pain disorders such as migraine. We have proposed that MAP kinases are important regulators of CGRP synthesis (Fig. 23.2). 5-HT₁ receptor agonists can repress CGRP secretion [118] and MAP kinase activation of the 18-bp CGRP enhancer [77, 119]. Based on a recent study, neuronal extracellular regulated kinase (ERK) MAP kinase inhibitors may be a promising strategy for attenuating allodynia in neuropathic pain [120]. A corollary approach will be to stimulate MAP kinase phosphatases. We have found that overexpression of MAP kinase phosphatase-1 lowers CGRP promoter activity [119]. These observations support the significance of future attempts to inhibit MAP kinase activation of CGRP synthesis. A novel approach to repress expression of CGRP synthesis is through posttranscriptional gene silencing. RNA interference (RNAi), a recently described biological process involved in posttranscriptional control of gene expression, could be manipulated to degrade CGRP mRNA, thus preventing its translation into the peptide. Although still far from application in humans, the therapeutic use of RNAi in neurological diseases is currently being explored, and several successes have been described in different animal models of human disease [121].

23.5.2 Potential for CGRP Antagonists and Migraine Drugs for Other Forms of Pain

The role of trigeminal CGRP is of particular significance due to the prevalence of painful craniofacial disorders. For migraine alone, it is estimated that 18 million Americans, including almost one in every five women, suffer an estimated 4 million

attacks every week [18, 19]. The chronic pain often precipitates other serious conditions such as depression [122, 123], and there is an enormous financial burden in the billions per year. A large number of people also suffer from other craniofacial pains, with estimates ranging from 5 to 12% of the population [124].

The events in other craniofacial pains, such as the neuropathic pain of trigeminal neuralgia, are even less understood than in migraine [125–127]. Elevated CGRP is associated with temporomandibular joint (TMJ) disorders [128–131] and eye conditions following laser or ocular surgery trauma [132]. Outside the craniofacial structures, elevated CGRP is also associated with other disorders, such as some forms of hypertension [133] and sepsis [134].

Due to the high incidence and generally poor efficacy of current treatments for trigeminal and other chronic-pain disorders, there is a need for improved therapeutic and preventative measures. Some of the drugs used to prevent migraines are also effective in the management of other forms of pain. Specifically, diabetic neuropathy and postherpetic neuralgia have been extensively studied, and TCAs and gabapentin, among others, are frequently used for symptomatic relief in these conditions [135, 136]. In contrast, carbamazepine, which is effective in the treatment of neuropathic pain [137] and is a first-line drug in the management of trigeminal neuralgia [87], has not shown efficacy in the treatment of migraines [138]. It is tempting to speculate that CGRP receptor antagonists might be beneficial in treating other conditions that involve elevated CGRP. Whether CGRP receptor antagonists will be effective in treating these conditions will be of intense basic and clinical interest in the upcoming years.

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INDEX

- Absorption pharmacokinetics
 - nicotine dependence and withdrawal, 543–544
- ABT431 compound
 - schizophrenia dopamine hypothesis, 373
- N*-Acetyl aspartate (NAA)
 - glutamate theory of schizophrenia
 - pathological evidence, 292
 - schizophrenia analysis, 256–257
 - schizophrenia dopamine hypothesis, 289
- N*-Acetylcysteine (NAC)
 - psychostimulant abuse and
 - cystine/glutamate transporter restoration, 576
- Acoustic startle
 - mouse anxiety models
 - neurosteroid effects, 147
- Acquired immunodeficiency syndrome (AIDS)
 - opiate addiction comorbidity with, 691–694
- Acute delivery systems
 - nicotine dependence and withdrawal therapy, 547–548
- Addictive disorders. *See also* Heroin
 - addiction; specific disorders, e.g.
 - Alcohol abuse
 - diagnostic criteria, 456–457
 - endocannabinoids, 675–676
 - γ -hydroxybutyric acid (GHB) and, 635–636
 - hallucinogens and, 639–640
 - nicotine dependence and withdrawal
 - pharmacology, 536–545
 - absorption pharmacokinetics, 543–544
 - bupropion, 549–550
 - cannabinoid antagonists, 550
 - distribution pharmacokinetics, 544
 - metabolism and elimination
 - pharmacokinetics, 544–545
 - nicotine reinforcement
 - neurosubstrates, 537–540
 - nicotinic acetylcholine receptors, 537
 - functional adaptations, 540–541
 - nicotinic antagonists, 548–549
 - nicotinic partial agonists, 548
 - nonnicotinic agents, 549–551
 - opioid antagonists, 550–551
 - overview, 545–546
 - pharmacokinetics, 543–545
 - public health policy and, 551–552
 - replacement medications, 546–548
 - tricyclic antidepressants, 550
 - withdrawal neurosubstrates, 541–543
 - opiate addiction
 - buprenorphine studies, 696–698
 - drug discovery survey, 699–700
 - epidemiology, 691–694
 - future research issues, 698–699
 - treatment statistics, 694–696

- pharmacotherapy
 - history of, 451–457
 - reward modulation/countermodulation and, 458–459
 - risk factors for addiction development, 457–458
- psychostimulant abuse
 - addiction therapy development, 585–586
 - chronic exposure-related neurobiology, 579–589
 - neurotransmitter/neuroendocrine systems, 580–584
 - signal transduction mechanisms and gene expression, 584–585
 - future research issues, 588–589
 - neuropharmacology, 569–579
 - γ -aminobutyric acid, 576–577
 - glutamate, 575–576
 - hypothalamic-pituitary-adrenal axis, 577–578
 - monoamines, 570–575
 - neuropeptides, 578–579
 - overview, 567–569
 - risk factors for, 455, 457–458
 - selective serotonin reuptake inhibitors
 - anxiety disorder therapy, 67–69
- Adenosine receptors
 - anxiety neurobiology and deficits in, 29–30
- A δ fibers
 - pain pathways, 711–712
- Adolescent patients
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 581–585
- Adrenergic receptors
 - antipsychotic drugs mechanisms, 426–427
 - psychostimulant abuse, 575
- Adrenocorticotropin hormone (ACTH)
 - anxiety neurobiology and, 17
 - nicotine dependence and withdrawal, 539–540
 - psychostimulant abuse, 577–578
 - chronic exposure and sensitization effects, 580–585
- Adult environmental effects
 - anxiety neurobiology, 35
- Affective flattening
 - in schizophrenia, 255–256
- Agonist agents
 - psychostimulant abuse therapy, 586–587
- AKT1 gene
 - dystrobrevin binding protein 1 (DTNBP1)
 - schizophrenia susceptibility, 348–349
- Alcohol
 - γ -hydroxybutyric acid (GHB) and, 634
 - neurosteroid effects and, 157–158
- Alcohol abuse
 - animal models
 - neuroanatomical substrates, 466–467
 - behavioral paradigms, 467–470
 - central nervous system circuitry, 470–471
 - novel substrates, 471
 - dopamine neuronal systems and substrates, 471–486
 - bed nucleus of stria terminalis, 473–475
 - dopaminergic regulation, early
 - research, 472–473
 - extracellular dopamine, 485–486
 - future research issues, 517–518
 - GABAergic interactions with, 486
 - lateral hypothalamus, 481–485
 - D₂ dopaminergic regulation, hypothesized mechanisms, 481–485
 - ventral pallidum, 476–480
 - dopaminergic regulation
 - hypothesized mechanisms, 478–480
- GABA_A benzodiazepine receptor
 - complex, molecular biology, 487–501
 - alcohol/modulator commonalities, 487–488
 - CA1/CA3 hippocampus, 511–516
 - efficacy of β CCT/3PBD modulation,
 - GABA_{A1,2,3,5} receptors, 499–501
 - future research issues, 517–518
 - GABA-DA interaction hypothesis, 496–498
 - ligand selectivity with GABA_{A1}
 - subunits, 498–499
 - microinjection studies, 505–506
 - naltrexone antagonist, 507–511
 - novel CNS GABAergic substrates, 498
 - oral administration, β CCT/3PBD
 - anxiety reduction, 510–511
 - vs. naltrexone, 507–511
 - probe applications, 488–493
 - site-specific microinjection, 493–496
 - subunit selectivity vs. intrinsic efficacy, 516–517

- systemic administration, 492–493, 503–504
 - ventral pallidum, 501–502
 - overview, 466
 - Alcohol-preferring rats (P rat line)
 - alcohol abuse studies
 - appropriate behavioral paradigms, 467–470
 - neuroanatomical controls, 469–470
 - reinforcer-specific controls, 469
 - central nervous system circuitry in, 470–471
 - characteristics of, 467
 - novel neuroanatomical substrates, 471
 - AlloTHDOC
 - plasma levels, 137–140
 - Alogia
 - in schizophrenia, 255–256
 - α -Amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)
 - benzodiazepine tolerance, 101
 - glutamate theory of schizophrenia
 - history of, 290–291
 - pathological evidence, 291–292
 - pharmacological evidence, 294–295
 - schizophrenia pharmacotherapy
 - drug development for, 392
 - Amino acid neurotransmission
 - anxiety disorder therapy, 72–75
 - anticonvulsants, 74–75
 - benzodiazepines, 73–74
 - Amino acid residues
 - benzodiazepines
 - GABA_A receptors, 106–108
 - D-Amino acid oxidase activator (DAO A)
 - schizophrenia candidate gene, 327
 - D-Amino acid oxidase (DAO)
 - schizophrenia candidate gene, 327
 - Amisulpiride
 - schizophrenia therapy
 - D₂/D₃ and D₄ receptor antagonism and regional specificity, 380
 - "AMPAkines"
 - glutamate theory of schizophrenia, 295
 - Amphetamine
 - chronic exposure and sensitization effects, 580–585
 - 3,4-methylenedioxymethamphetamine
 - reinforcement of, 615–616
 - monoamine neuropharmacology, 571–575
 - neuropeptide pharmacology and, 579
 - d*-Amphetamine
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - Amygdala. *See also* Extended amygdala (EA)
 - anxiety disorders and, 13–15
 - Anandamide
 - appetite regulation and, 671–672
 - endocannabinoid ligands, 663–664
 - pain management and, 667–668
 - pharmacology, 665–675
 - Anesthesia
 - neuroactive steroid effects and, 158–159
 - Animal models. *See also* Mouse studies
 - alcohol-motivated behaviors
 - neuroanatomical substrates, 466–467
 - behavioral paradigms, 467–470
 - central nervous system circuitry, 470–471
 - novel substrates, 471
 - anxiety neurobiology
 - anxiety-like behavior in, 10–12
 - brain imaging studies, 14–15
 - early-life environmental effects, 33–34
 - rodent models, 6–9
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity, 620–621
 - behavioral effects, 630–631
 - neurosteroid effects, 142–152
 - acoustic/fear-potentiated startle, 147
 - defensive burying behavior, 149–150
 - elevated-plus maze, 143–145
 - Geller/Seifter and Vogel conflict tests, 145–146
 - light-dark box, 146–147
 - mild mental stress models and social isolation, 151–152
 - mirrored chamber, 147–148
 - modified forced-swim test, 150–151
 - open-field activity, 148–149
 - separation-induced ultrasonic vocalizations, 150
 - obsessive-compulsive disorders (OCD), 224
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 581–585
 - schizophrenia, 263–265
 - transient receptor potential V1 (TRPV1) receptors, 736
- Anticonvulsants
 - anxiety disorder therapy, 74–75

- migraine management, 766–767
- Δ^9 -tetrahydrocannabinol as, 672–673
- Antidepressants
 - anxiety disorder therapy, 64–70
 - beta blockers, 71
 - mirtazapine, 70
 - monoamine oxidase inhibitors, 70
 - selective serotonin reuptake inhibitors, 66–69
 - serotonin and noradrenaline reuptake inhibitors, 69
 - serotonin receptor agonists, 70–71
 - tricyclic antidepressants, 69–70
- migraine management, 767
- nicotine dependence and withdrawal therapy
 - bupropion, 549–550
- tricyclic
 - nicotine dependence and withdrawal therapy, 550
- Antiemetics
 - migraine management, 765
- Antihistamines
 - anxiety disorder therapy, 75
- Antinociception
 - cannabinoid analgesics and, 668
- Antipsychotics
 - anxiety disorder therapy, 71–72
 - atypical agents, mechanisms of action
 - adrenergic mechanisms, 426–427
 - atypical antipsychotics
 - 5-HT_{2A} receptor, 418–421
 - cholinergic mechanisms, 429–430
 - clozapine regimens
 - dopamine D₂ receptor blockade, 412–415
 - glutamatergic mechanisms, 427–429
 - neurogenesis, 430
 - neurokinin 3 receptors, 430
 - partial dopamine agonists, 416–417
 - serotonin receptor 5-HT_{1A}-5-HT_{2A} interactions, 424–425
 - serotonin receptor 5-HT_{2A}-5-HT_{2C} interactions, 422–424
 - serotonin receptor 5-HT₆, 425–426
 - serotonin receptors, 417–418
 - serotonin release, 426
 - serotonin receptor 5-HT_{2A} blockade
 - cortical dopamine efflux and cognitive function, 421–422
 - extrapyramidal function, 422
 - substituted benzamides
 - D₁, D₃, and D₄ receptors, 415–416
 - table of drugs, 413
 - typical vs. atypical drugs, 411–412
- metabolic syndrome with, 254
- obsessive-compulsive disorders
 - neuropharmacology, 229–234
 - symptom induction from, 234
- schizophrenia therapy
 - first-generation (conventional) agents, 383
 - glutamate theory of schizophrenia, 294–295
 - history of, 370–371
 - hypothesized mechanisms of action, 376–382
 - D₁ receptors, 380–381
 - D₂/D₃ and D₄ antagonism and regional specificity, 380
 - D₂ occupancy thresholds and rapid dissociation, 378–380
 - D₂ receptor occupancy, 376–378
 - dopamine release, 381
 - NMDA receptor function, 381
 - synthesis reactions, 381–382
 - negative affect remediation, 255–256
 - psychosis management, 254
 - safety and tolerability, 387–388
 - second-generation (atypical) agents, 383–387
- Anxiety and anxiety disorders
 - alcohol abuse and
 - β CCT/3PBC anxiety reduction efficacy, 510–511
 - clinical management, 60–61
 - corticotropin-releasing factor receptor antagonists
 - therapeutic potential of, 196–198
 - depression and
 - pharmacotherapy, 80
 - diagnostic criteria, 9–12, 61–62
 - 3,4-methylenedioxymethamphetamine
 - effects, 630–631
 - neuroactive steroids
 - alcohol effects, 157–159
 - animal models, 142–152
 - behavioral effects, 142
 - brain and peripheral sources, 135–137
 - chemistry and pharmacology, 135–142
 - enantiomeric selectivity, 141–142
 - GABA_A receptors and ligand-gated ion channels, 137–141

- HPA axis function, 154–156
- overview, 134–135
- stress-induced behaviors, 154–155
- neurobiology of
 - basic principles, 4
 - early-life environmental effects, 33–37
 - fear/anxiety circuits, 13–15
 - genetic susceptibility, 18–19
 - intracellular regulators, 30–33
 - knockout mice
 - neuronal messenger alterations, 24–26
 - neurotransmitter receptor deficits and CRH proteins, 26–30
 - mouse genetics studies, 19–24
 - neurotransmitter systems and neuronal messengers, 15–18
 - psychological traits
 - continuous expression of normal personality, 9–10
 - genetic basis of, 4–6
 - mouse behavior extrapolation studies, 6–9
- pharmacotherapy
 - anxiolytic drugs, 62–75
 - amino acid neurotransmission, 72–75
 - anticonvulsants, 74–75
 - antidepressants, 64–70
 - antihistamines, 75
 - antipsychotics, 71–72
 - benzodiazepines, 73–74
 - beta-blockers, 71
 - lithium, 75
 - monoamine neurotransmission, 63–72
 - serotonin receptor agonists, 70–71
 - depressive disorders, 80
 - future research issues, 81
 - generalized anxiety disorder, 77
 - obsessive-compulsive disorder, 77–78
 - overview, 60
 - panic disorder/agoraphobia, 78
 - phobias, 78, 80
 - posttraumatic stress disorder, 79
 - social anxiety disorder, 79–80
 - treatments chart, 76
- "Anxiety-related pathways"
 - anxiety neurobiology and, 36
- Anxiolytic drugs
 - anxiety disorder pharmacotherapy, 62–75
 - amino acid neurotransmission, 72–75
 - anticonvulsants, 74–75
 - antidepressants, 64–70
 - antihistamines, 75
 - antipsychotics, 71–72
 - benzodiazepines, 73–74
 - beta-blockers, 71
 - lithium, 75
 - monoamine neurotransmission, 63–72
 - serotonin and noradrenaline reuptake inhibitors, 69
 - serotonin receptor agonists, 70–71
 - tricyclic antidepressants, 69–70
 - corticotropin-releasing factor receptor antagonists
 - future research issues, 198–199
 - nonpeptide ligands, 195–196
 - overview, 177–179
 - peptide ligand pharmacology, 185–195
 - ligand binding mechanisms, 190–195
 - receptor/ligand family structure, 179–185
 - ligand properties, 180–181
 - receptor subtypes and distribution, 181–185
 - therapeutic potential, 196–198
- Appetite regulation
 - cannabinoid receptors and, 670–672
- Arachidonic acid derivatives
 - endocannabinoid ligands, 663–664
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 732–733
- N*-Arachidonoyldopamine
 - endocannabinoid ligands, 663–664
- Arachidonylethanolamide
 - cannabinoid receptors and, 661–662
- O*-Arachidonylethanolamine
 - endocannabinoid ligands, 663–664
- Arousal
 - autonomic arousal
 - anxiety neurobiology, 4
 - animal anxiety-like behavior, 10–12
- Asenapine
 - schizophrenia therapy, 389
- Assembly mechanisms
 - GABA_A receptors
 - benzodiazepines, 108–110
- Astressin
 - corticotropin-releasing factor receptor antagonists
 - ligand binding mechanisms, 191–195

- Attention-deficit hyperactivity disorder (ADHD)
 - obsessive-compulsive disorder
 - comorbidity, 216
 - psychostimulant therapy, 568–569, 587
 - chronic exposure and sensitization effects, 581–585
- Attention deficits
 - schizophrenia
 - animal models, 263–264
- Augmentation strategies
 - obsessive-compulsive disorders
 - neuropharmacology, 225, 229–234
- Autonomic arousal
 - anxiety neurobiology, 4
 - animal anxiety-like behavior, 10–12
- Autoreceptors
 - partial dopamine agonists, 416–417
- Avoidance behaviors
 - anxiety neurobiology, 4
 - animal anxiety-like behavior, 10–12
- Avolition
 - in schizophrenia, 255–256
- Baclofen
 - psychostimulant abuse therapy, 588
- Basal ganglia-thalamic-frontal loops
 - obsessive-compulsive disorders
 - neuropharmacology, 221–222
- BC1 RNA
 - anxiety neurobiology and, 32
- Bed nucleus of stria terminalis (BST)
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 473–475
- Behavioral inhibition/activity
 - alcohol abuse
 - animal models
 - neuroanatomical substrates, 466–467
 - behavioral paradigms, 467–470
 - central nervous system circuitry, 470–471
 - novel substrates, 471
 - dopamine neuronal systems and substrates, 471–486
 - bed nucleus of stria terminalis, 473–475
 - dopaminergic regulation, early research, 472–473
 - extracellular dopamine, 485–486
 - future research issues, 517–518
 - GABAergic interactions with, 486
 - lateral hypothalamus, 481–485
 - ventral pallidum, 476–480
- Benzamides
 - D₁, D₃, and D₄ receptors, 415–416
- Benzodiazepines (BZs). *See also*
 - GABA_A benzodiazepine receptor complex
 - anxiety disorder therapy, 73–74
 - flunitrazepam, 637
 - future research, 116–117
 - GABA_A benzodiazepine receptor complex
 - alcohol abuse studies, 487–501
 - alcohol/modulator commonalities, 487–488
 - CA1/CA3 hippocampus, 511–516
 - efficacy of β CCT/3PBD modulation, GABA_{A1,2,3,5} receptors, 499–501
 - future research issues, 517–518

- GABA-DA interaction hypothesis, 496–498
- ligand selectivity with GABA_{A1} subunits, 498–499
- microinjection studies, 505–506
- naltrexone antagonist, 507–511
- novel CNS GABAergic substrates, 498
- oral administration, β CCT/3PBD
 - anxiety reduction, 510–511
 - vs. naltrexone, 507–511
- probe applications, 488–493
- site-specific microinjection, 493–496
- subunit selectivity vs. intrinsic efficacy, 516–517
- systemic administration, 492–493, 503–504
- ventral pallidum, 501–502
- GABA_A receptors
 - assembly, clustering, and surface expression, 108–110
 - binding pocket, 105–108
 - brain function diversity, 110–112
 - functional diversity, knockout/knockin models, 112–114
 - single-cell response modulation, 99–100
 - structure and function, 93–96
 - subunit/subtype diversity, 103–104
- 3,4-methylenedioxymethamphetamine
 - effects on, 628
- receptor ligand pharmacology, 97–102
 - endogenous site, 98–99
 - metabolism functions, 101–102
 - single-cell GABA response modulation, 99–100
 - therapeutic action, 97–98
 - tolerance and dependence
 - characteristics, 100–101
 - structure-activity relationships, 114–116
 - structure and function, 93–96
- Beta-blockers
 - anxiety disorder therapy, 71
 - migraine management, 767
- BIBN4096BS compound
 - calcitonin gene-related peptide sites and migraine therapy targeting, 762–764
- Bicuculline
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex, 487–488
 - site-specific microinjection techniques, 494–496
- Bifeprunox
 - schizophrenia therapy, 389
- Binding pocket structures
 - benzodiazepines
 - GABA_A receptors, 105–108
- Biochemical markers
 - in schizophrenia, 260–262
- Biochemistry
 - 3,4-methylenedioxymethamphetamine
 - effects, 616–619
- Biogenesis of lysosome-related organelles complex-1 (BLOC1)
 - dystrobrein binding protein 1 (DTNBP1)
 - molecular interactions, 353
- Biological mechanisms
 - of schizophrenia, 256–263
- Blood alcohol concentration (BAC) levels
 - alcohol abuse studies in alcohol-preferring rats, 468–470
- Body temperature
 - 3,4-methylenedioxymethamphetamine
 - effects on, 617–618
 - neurotoxicity and, 624–625
- Botulinum toxin (Botox)
 - migraine management, 768
- Brain
 - anxiety disorder-related regions of, 13–15
 - benzodiazepine distribution, 110–112
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity
 - biochemical and functional changes, 631–632
 - neuroactive steroid sources, 135–137
 - neuroanatomical control regions
 - alcohol abuse studies in
 - alcohol-preferring rats, 469–470
 - circuitry systems, 470–471
 - psychostimulant abuse, 574–575
- Brain-derived neurotrophic factor (BDNF)
 - anxiety neurobiology, 4
 - genetic susceptibility studies, 18–19
 - knockout mice studies, 25–26
 - receptor deficits and, 29–30
 - schizophrenia therapy
 - serotonin receptor 5-HT_{2A} receptor and, 421–422
- Brain imaging studies
 - obsessive-compulsive disorders, 217–222
 - animal models, 224

- neuropharmacological implications, 220–222
- schizophrenia, 256–260
- Buprenorphine
 - opiate addiction therapy
 - comparative efficacy studies, 697–698
 - implementation issues and pilot studies, 698
 - regulatory approval studies, 696–697
- Bupropion
 - nicotine dependence and withdrawal therapy, 549–550
- Buspirone
 - anxiety disorder therapy, 70–71
 - opiate addiction therapy, 699
- Butyrophenones
 - schizophrenia therapy
 - clinical profile, 383
- CA1/CA3 hippocampal fields
 - alcohol abuse studies
 - GABA_{A5} receptor probes, 511–516
- Calcitonin gene-related peptide (CGRP)
 - migraine therapy, 759–763
 - injection techniques, 763
 - neurovascular model, 759–760
 - receptor antagonist therapeutic efficacy, 763–764
 - synthesis and actions, 760–761
 - targeted repression therapy, 768
 - trigeminovascularity sites, 761–763
- pain management applications, 768–769
- Calcitonin receptor-like receptor (CLR)
 - calcitonin gene-related peptide sites and migraine therapy targeting, 762
- Calcium/calmodulin-dependent protein kinase II (CaMK II)
 - anxiety neurobiology and, 30–33
 - benzodiazepine tolerance, 101
- Calcium channel blockers
 - migraine management, 767–768
 - vanilloid receptors and, 728–729
- Calmodulin kinase II
 - transient receptor potential V1 (TRPV1) receptor sensitization, 735
- cAMP response element binding (CREB) protein
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 585
- Cannabinoid hypothesis of schizophrenia
 - pharmacotherapy, 394
- Cannabinoid receptors. *See also* Marijuana
 - analgesic properties, 666–668
 - cannabinoid₁ (CB-1) receptor
 - addictive disorders
 - modulator therapy and, 458–459
 - antagonist/agonist medication
 - development, 700
 - anxiety neurobiology and
 - deficits in, 29–30
 - appetite regulation, 670–672
 - cognition and, 669–670
 - convulsant effects, 672–673
 - emesis modulation, 673–675
 - identification of, 661–662
 - nicotine dependence and withdrawal therapy
 - antagonist agents, 550
 - pain management and, 667–668
 - prenatal development and, 664–665
 - signaling mechanisms, 662–663
 - cannabinoid₂ (CB-2) receptor
 - emesis modulation, 674–675
 - pain management and, 668–669
 - signaling mechanisms, 662–663
 - cognition and, 669–670
 - convulsant effects, 672–673
 - emesis modulation, 673–675
 - future research on, 676
 - reward, tolerance, and dependence, 675–676
 - in schizophrenia
 - animal model, 265
 - signaling mechanisms, 662–663
- CAPON gene
 - schizophrenia molecular genetics, 328
- Capsaicin
 - cloning of, 727–729
 - transient receptor potential V1 (TRPV1) receptor expression, 730–732
- Carbamazepine
 - anxiety disorder therapy, 75
- β -Carboline-3-carboxylate-*t*-butyl ester
 - alcohol abuse studies
 - anxiety reduction with, 510–511
 - GABA_{A1,2,3,5} receptor subunit
 - modulation, 499–501
 - GABA_{A1} receptor subunit selectivity, 498–499
 - microinjection techniques, 505–507
 - oral administration, 507–511
 - systemic administration, 503–504

- β -Carboline antagonist ZK 93426
 - alcohol abuse studies
 - GABA_{A5} receptor specificity, 514–516
 - GABA_A benzodiazepine receptor complex, 490–493
 - GABAergic modification of dopamine agonists, 497
- Cardiovascular system
 - γ -hydroxybutyric acid (GHB) effects, 634
- Caspase-1
 - 3,4-methylenedioxymethamphetamine effects on, 628
- Catechol-*O*-methyl transferase (COMT)
 - anxiety neurobiology and
 - knockout mice studies, 24–26
 - obsessive-compulsive disorders
 - metabolism studies, 223
 - schizophrenia
 - dopamine hypothesis, 373
 - genetic evidence, 286–287
 - genetics studies, 286–287
 - imaging studies, 285–286
 - molecular genetics
 - chromosomal abnormalities, 328–329
 - susceptibility gene identification, 347–348
 - tolcapone targeting of, 389
- Cell-cell interactions
 - anxiety neurobiology
 - neuronal deficits and, 30
- Cell membrane-associated proteins
 - anxiety neurobiology and
 - mouse studies, 19, 21
- Central nervous system (CNS)
 - alcohol abuse studies in alcohol-preferring rats
 - circuitry systems, role of, 470–471
 - control substrates, 469–470
 - γ -hydroxybutyric acid (GHB) effects, 634
 - pain pathways, 712–714
 - schizophrenia and, 260–262
- Cerebrospinal fluid (CSF)
 - corticotropin-releasing factor receptor antagonists
 - effects on, 177–179
 - schizophrenia dopamine hypothesis and, 288–289
- C fibers
 - pain pathways, 711–712
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 729
- Chemotherapy
 - emesis with
 - cannabinoid modulation of, 674–675
- Cholecystokinin
 - anxiety neurobiology and, 17
 - pain management and, 716–717
- Cholinergic neurotransmitters
 - atypical antipsychotic mechanisms, 429–430
 - schizophrenia neurochemistry and, 261–262
 - schizophrenia pharmacotherapy, 393–394
- Chromosomal abnormalities
 - schizophrenia molecular genetics, 328–331
 - catechol-*O*-methyltransferase, 328–329
 - DISC1 gene, 330–331
 - PRODH candidate gene, 329–330
 - ZDHC8 candidate gene, 330
- Chromosome mapping
 - anxiety neurobiology
 - mouse studies, 19, 23
 - schizophrenia
 - susceptibility gene identification, 346–351
- CL218,872
 - alcohol abuse studies
 - GABA_{A1} receptor subunit selectivity, 499
- Clomipramine
 - obsessive-compulsive disorder therapy, 69–70
- Clonazepam
 - obsessive-compulsive disorder therapy, 234
- Clonidine
 - schizophrenia pharmacotherapy, 393
- Clozapine
 - dopamine D₂ receptor blockade, 412–415
 - mechanisms of action
 - cholinergic mechanisms, 429–430
 - serotonin receptor 5-HT_{2A}, 418–421, 420–421
 - serotonin receptor 5-HT₆ and, 425–426
 - serotonin receptor 5-HT/D₂ hypothesis, 417–418
 - schizophrenia therapy
 - brain-derived neurotrophic factor and, 421–422
 - cholinergic receptor targeting, 393–394
 - clinical profile, 387
 - dopamine D₂ receptor blockade, 412–415

- history of, 370
 - safety and tolerability, 388–389
 - schizophrenia-related psychosis, 254
- Clusters
 - GABA_A receptors
 - benzodiazepines, 108–110
- Cocaine
 - abuse
 - 3,4-methylenedioxymethamphetamine
 - reinforcement of, 615–616
 - monoamine neuropharmacology, 570–575
 - therapy developments for, 585–588
 - chronic exposure and sensitization effects, 580–585
 - neuropeptide pharmacology and, 578–579
- Cocaine- and amphetamine-regulated transcript (CART) peptides
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 585
- Cognitive behavior therapy (CBT)
 - obsessive-compulsive disorders, 219–220
- Cognitive function
 - cannabinoid receptors and, 669–670
 - 3,4-methylenedioxymethamphetamine and, 630–631
 - nicotine dependence and withdrawal
 - withdrawal neurosubstrates, 541–543
 - schizophrenia, 254–255
 - animal model, 263–264
 - glutamatergic receptors, 428–429
 - neural network analysis, 259
 - serotonin receptor 5-HT_{2A} receptor enhancement, 421–422
- Comorbid conditions
 - addictive disorders vulnerability with, 457–458
 - obsessive-compulsive disorders, 216
 - opiate addiction, 691–694
- Compensatory mechanisms
 - dopamine hypothesis of schizophrenia, 284–285
- Computed tomography (CT)
 - obsessive-compulsive disorders
 - brain imaging studies, 217–218
 - schizophrenia analysis, 256
- Conditioned conflict tests
 - anxiety neurobiology
 - emotionality studies in mice, 8–9
- Conditioned fear paradigms
 - emotionality studies in mice, 8–9
- Continuous performance test (CPT)
 - schizophrenia
 - animal models, 263–264
- Cortical dopamine
 - schizophrenia dopamine hypothesis and, 287–288
 - serotonin receptor 5-HT_{2A} receptor enhancement, 421–422
- Corticosterone
 - psychostimulant abuse, 577–578
- Corticostriatal-thalamic-cortical loops
 - integrated glutamate/dopamine
 - hypotheses of schizophrenia
 - neurochemistry and, 374–376
- Corticotropin-releasing factor (CRF)
 - alcoholol abuse studies
 - in alcohol-preferring rats, 471
 - neurosteroid effects
 - acoustic/fear-potentiated startle, 147
 - nicotine dependence and withdrawal, 539–540
 - withdrawal substrate specificity, 543
 - opiate dependence therapy and, 699
 - psychostimulant abuse, 577–578
 - chronic exposure and sensitization effects, 580–585
- Corticotropin-releasing factor receptor
 - antagonists
 - anxiolytic applications
 - future research issues, 198–199
 - nonpeptide ligands, 195–196
 - overview, 177–179
 - peptide ligand pharmacology, 185–195
 - ligand binding mechanisms, 190–195
 - receptor/ligand family structure, 179–185
 - ligand properties, 180–181
 - receptor subtypes and distribution, 181–185
 - therapeutic potential, 196–198
- Corticotropin-releasing hormone (CRH)
 - anxiety neurobiology and, 17
 - knockout mice studies, 25–26
 - receptor deficits, 28–30
- Cortisol
 - nicotine dependence and withdrawal, 539–540
 - withdrawal substrate specificity, 543
- Cotinine
 - nicotine dependence and withdrawal
 - metabolism and elimination
 - pharmacokinetics, 544–545

- Countermodulation therapy
 - addictive disorders, 458–459
- CP-154,526 antagonist
 - opiate dependence therapy and, 699
- CRF₁ receptor
 - corticotropin-releasing factor receptor
 - antagonists, 181–185
 - anxiety/depression therapeutic potential
 - and, 196–198
 - ligand binding mechanisms, 190–195
 - nonpeptide ligands, 195–196
 - peptide ligand pharmacology, 185–195
- CRF_{2(a)} receptor
 - corticotropin-releasing factor receptor
 - antagonists, 182–185
- CRF_{2(b)} receptor
 - corticotropin-releasing factor receptor
 - antagonists, 182–185
- CRF₂ receptor
 - corticotropin-releasing factor receptor
 - antagonists, 182–185
 - ligand binding mechanisms, 190–195
 - peptide ligand pharmacology, 185–195
- CX516 compound
 - schizophrenia pharmacotherapy, 392
- Cyclic adenosine monophosphate (cAMP)
 - corticotropin-releasing factor receptor
 - antagonists
 - CRF receptor stimulation, 186–195
 - psychostimulant abuse
 - chronic exposure and sensitization
 - effects, 584–585
- Cyclic adenosine monophosphate (cAMP)-responsive nuclear factors
 - anxiety neurobiology and, 32
- CYP1A2 enzyme
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 622
- CYP2A6 enzyme
 - nicotine dependence and withdrawal
 - metabolism and elimination
 - pharmacokinetics, 545
- Cystine/glutamate transporters
 - psychostimulant abuse and, 576
- Cytochrome P450 enzymes
 - benzodiazepine receptor ligand
 - metabolism, 102
 - selective serotonin reuptake inhibitors,
 - 68–69
- Cytogenetic abnormalities
 - schizophrenia
 - susceptibility gene identification,
 - 346–351
- Cytokines
 - anxiety neurobiology, 16–18
 - knockout mice studies, 26
 - 3,4-methylenedioxymethamphetamine
 - effects on, 627–628
- D₁ receptor
 - alcohol abuse studies
 - bed nucleus of stria terminalis system,
 - 475
 - lateral hypothalamus, dopaminergic
 - regulation by, 481–485
 - ventral pallidum pathways, 477–480
 - psychostimulant abuse
 - monoamine neuropharmacology,
 - 571–575
 - schizophrenia dopamine hypothesis
 - cortical vs. striatal dopamine, 288
 - history, 284–285
 - imaging evidence, 286–287
 - schizophrenia pharmacotherapy
 - antipsychotic agents, 380–381
 - schizophrenia therapy
 - agonist and antagonists, 390
 - substituted benzamides, 415–416
- D₂/D₃ receptors
 - clozapine D₂ receptor blockade, 414–415
 - partial dopamine agonists, 416–417
 - psychostimulant abuse therapy,
 - 586–587
 - selective antagonism
 - schizophrenia therapy, 380
 - substituted benzamides, 416
- D₂ receptor
 - alcohol abuse studies
 - lateral hypothalamus, dopaminergic
 - regulation by, 481–485
 - ventral pallidum pathways, 477–480
 - clozapine blockade, 412–415
 - psychostimulant abuse
 - chronic exposure and sensitization
 - effects, 582–585
 - monoamine neuropharmacology,
 - 571–575
 - schizophrenia dopamine hypothesis
 - cortical vs. striatal dopamine, 288
 - history, 284–285, 371–373
 - imaging evidence, 285–286
 - pathological evidence, 285
 - pharmacological evidence, 287

- schizophrenia pharmacotherapy
 - antipsychotic occupancy and effect, 376–378
 - High 5-HT_{2A} vs., 378
 - occupancy thresholds and rapid dissociation, 378–380
 - subtherapeutic occupancy time, 382
- D₃ receptor
 - schizophrenia dopamine hypothesis
 - genetic evidence, 286–287
 - pathological evidence, 285
 - pharmacological evidence, 287
 - schizophrenia therapy
 - antagonist development, 390
 - substituted benzamides, 415–416
- D₄ receptor
 - schizophrenia dopamine hypothesis
 - pathological evidence, 285
 - pharmacological evidence, 287
 - schizophrenia therapy
 - antagonism and regional specificity, 380
 - antagonist development, 390
 - substituted benzamides, 415–416
- Deep brain stimulation (DBS)
 - obsessive-compulsive disorders, 236–237
- Defensive burying behavior
 - animal anxiety models
 - neurosteroid effects, 149–150
- Dehydroepiandrosterone
 - schizophrenia therapy, 394
- Dehydroepiandrosterone sulfate (DHEAS)
 - in brain, 137
 - schizophrenia therapy, 394
- Delta opioid receptors
 - structure and function, 751
- Dependency risk
 - endocannabinoids, 675–676
- Depolarization inactivation
 - schizophrenia therapy
 - D₂ receptor occupancy and effect, 376–378
- Depression
 - anxiety symptoms with
 - pharmacotherapy, 80
 - corticotropin-releasing factor receptor
 - antagonists
 - therapeutic potential of, 196–198
 - neuroactive steroid interactions, 156–157
 - stress-induced behavior
 - neuroactive steroids, 156–157
- Desensitization
 - transient receptor potential V1 (TRPV1) receptors, 735–736
- Designer drugs. *See also* specific drugs, e.g. 3,4-Methylenedioxymethamphetamine (MDMA)
 - overview, 614
- Developmental genes
 - obsessive-compulsive disorders, 223–224
- Diagnostic criteria
 - anxiety disorders, 9–12, 61–62
- Diazepam binding inhibitor (DBI)
 - benzodiazepine ligands, 98–99
- 3,4-Dihydroxyamphetamine (HHA)
 - 3,4-methylenedioxymethamphetamine neurotoxicity and, 622
- 3,4-Dihydroxy-L-phenylalanine (DOPA)
 - schizophrenia dopamine hypothesis, 372–373
- Dimerization
 - opioid receptors, 752
- DISC1 gene
 - schizophrenia molecular genetics, 330–331
 - molecular interactions, 351–352
 - susceptibility identification, 349–350
- Discontinuation syndrome
 - selective serotonin reuptake inhibitors, 67–69
- Distribution kinetics
 - nicotine dependence and withdrawal, 544
- DOI
 - hallucinogens and, 639
 - schizophrenia therapy
 - serotonin receptor 5-HT_{2A}, 420–421
- Donepezil
 - schizophrenia pharmacotherapy, 393–394
- Dopadecarboxylase
 - schizophrenia dopamine hypothesis, 372–373
- Dopamine agonist
 - alcohol abuse studies
 - GABAergic modification, 496–498
 - partial agonists, 416–417
 - psychostimulant abuse therapy, 586–587
 - in schizophrenia
 - animal models, 264
- Dopamine hypothesis of schizophrenia
 - cortical vs. striatal dopamine, 287–289
 - genetic evidence, 286–287
 - glutamate theory consolidated with, 295–296
 - history, 284–285

- imaging evidence, 285–286
- neurochemistry and, 371–373
- pathological evidence, 285
- pharmacological evidence, 287
- postmortem studies, 344
- Dopamine neurotransmitters
 - addictive disorders
 - reward therapy and, 458–459
 - anxiety disorder therapy
 - antipsychotics, 71–72
 - anxiety neurobiology, 15–17
 - receptor deficits, 29–30
 - 3,4-methylenedioxymethamphetamine (MDMA)
 - receptor/transporter effects, 617
 - reinforcing properties, 614–616
 - nicotine dependence and withdrawal
 - withdrawal substrates, 542–543
 - obsessive-compulsive disorders, 223
 - psychostimulant abuse, 569–575
 - neuropeptide pharmacology, 579
 - neuropharmacology, 570–575
 - schizophrenia and, 260–262
 - COMT catabolism and, 328
 - drug targeting innovations, 389
- Dopaminergic receptors
 - alcohol abuse studies
 - neuronal systems and substrates, 471–486
 - bed nucleus of stria terminalis, 473–475
 - dopaminergic regulation, early
 - research, 472–473
 - extracellular dopamine, 485–486
 - future research issues, 517–518
 - GABAergic interactions with, 486
 - lateral hypothalamus, 481–485
 - D₂ dopaminergic regulation, hypothesized mechanisms, 481–485
 - ventral pallidum, 476–480
 - hypothesized mechanisms, 478–480
- alcohol abuse studies
 - in alcohol-preferring rats, 471
- anxiety neurobiology and receptor deficits, 29–30
- nicotine dependence and withdrawal
 - nicotine reinforcement substrates, 538–540
- psychostimulant abuse
 - chronic exposure and sensitization effects, 582–585
 - glutamatergic interactions, 575–576
 - monoamine neuropharmacology, 571–575
 - schizophrenia molecular genetics
 - functional candidate genes, 331–332
- Dopamine transporter
 - catechol-*O*-methyl transferase
 - as schizophrenia susceptibility gene, 347–348
- inhibitors
 - psychostimulant therapy targeting, 587–588
- obsessive-compulsive disorders
 - abnormalities, 219–220
- psychostimulant abuse
 - monoamine neuropharmacology, 573–575
- schizophrenia
 - dopamine hypothesis and, 285
- Dorsolateral prefrontal cortex (DLPFC)
 - schizophrenia dopamine hypothesis, 373
- DRD2/DRD3 receptors
 - schizophrenia molecular genetics, 331–332
 - catechol-*O*-methyl transferase
 - susceptibility gene, 347–348
- Drug abuse
 - dopaminergic receptor systems and substrates
 - bed nucleus of stria terminalis system, 474–475
 - rewarding/reinforcing effects in, 459
 - stress-induced response
 - neuroactive steroid effects and, 159–160
- Dyadic encounters
 - schizophrenia
 - animal models, 264
- Dynorphins
 - structure and classification, 747
- Dysbindin
 - dystrobrevin binding protein 1 (DTNBP1)
 - molecular interactions, 353
 - schizophrenia candidate gene, 326, 348–349
- Dystrobrevin binding protein 1 (DTNBP1)
 - schizophrenia candidate gene, 325–326
 - chromosome mapping, 346
 - chromosome identification of, 348–349
 - functional implications, 332–333
 - molecular interactions, 352–353
- Dystrophin-associated glycoprotein complex (DCG)

- dystrobrevin binding protein 1
 - identification
 - schizophrenia candidate gene, 348–349
- Electroencephalography (EEG)
 - schizophrenia analysis, 256
- Elevated-plus maze (EPM)
 - animal anxiety-like behavior and, 12
 - neurosteroid effects, 143–145
 - emotionality studies in mice, 7–9
- Elevated-zero maze (EZM)
 - emotionality studies in mice, 8–9
- Elimination mechanisms
 - nicotine dependence and withdrawal, 544–545
- Emesis
 - antiemetics
 - migraine management, 765
 - cannabinoid receptors modulation of, 673–675
- Emotionality studies
 - anxiety neurobiology
 - rodent models, 6–9
- Enantiomeric selectivity
 - neuroactive steroids, 141–142
- Endocannabinoid system, 662–665
 - cannabinoid receptors and signaling, 662–663
 - physiology and pharmacology, 665–675
 - prenatal developmental effects, 664–665
 - reward, tolerance and dependence mechanisms, 675–676
 - synthesis and metabolism, 663–664
- Endogenous opioids
 - classification, 746–748
- Endogenous sites
 - benzodiazepine ligands, 98–99
- β -Endorphins
 - structure and classification, 747–748
- Enkephalin neurons
 - nicotine dependence and withdrawal, 540
- Enkephalins
 - structure and function, 746–747
- Enthoprotin
 - schizophrenia molecular genetics, 328
- Environmental factors
 - addictive disorders vulnerability, 457–458
 - anxiety neurobiology
 - early-life experience, 33–34
- Epilepsy
 - endocannabinoids and, 672–673
- ErbB3 gene
 - NRG1 molecular interaction, 353–355
 - regulator of G-protein signaling 4
 - schizophrenia candidate gene, 328
- Ergots
 - migraine management, 765
- Ethanol
 - neuroactive steroid effects and, 158–159
- Eticlopride
 - alcohol abuse studies
 - lateral hypothalamus, effects on, 481–485
 - ventral pallidum receptor blockade, 478
- Excitatory amino acid transporters (EAATs)
 - glutamate theory of schizophrenia
 - pathological evidence, 292
 - schizophrenia genetics
 - GRM3 gene, 349
- Extended amygdala (EA)
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 473–474
 - GABA_A benzodiazepine receptor
 - complex manipulation
 - site-specific microinjection studies, 493–496
- Extracellular dopamine
 - alcohol abuse studies
 - dopaminergic regulation mechanisms, 485–486
- Extrapyramidal symptoms (EPS)
 - atypical antipsychotic drugs, 411–412
 - clozapine D₂ receptor blockade, 412–415
 - schizophrenia therapy
 - antipsychotics and, 370–371
 - D₂ receptor occupancy and effect, 376–378
 - D₂ receptor occupancy thresholds and rapid dissociation, 379–380
 - first-generation antipsychotics, 383
 - second-generation antipsychotics, 383–388
 - serotonin receptor 5-HT_{2A} blockade, 422
- Extroversion *vs.* introversion (E trait)
 - anxiety neurobiology, 5–6
- Face validity
 - animal anxiety-like behavior and, 12
- Fatty acid amide hydrolase (FAAH)
 - convulsant effects of, 672–673
 - emesis modulation, 674–675

- endocannabinoid ligands, 664
- endocannabinoid physiology and, 666
- Fear/anxiety circuits
 - anxiety neurobiology, 13–15
- Fear-potentiated startle
 - mouse anxiety models
 - neurosteroid effects, 147
- Feverfew
 - migraine management, 768
- FEZ1 protein
 - DISC1 schizophrenia candidate gene
 - molecular interactions, 351–352
- First-generation antipsychotics (FGAs)
 - schizophrenia therapy
 - clinical profiles, 383
 - D₂ receptor occupancy and effect, 376–378
 - history of, 370–371
 - NMDA receptor antagonists, 381
 - second-generation antipsychotic comparisons, 384–387
- Fixed-ratio (FR) schedule
 - alcohol abuse studies in alcohol-preferring rats, 469
- Flumazenil
 - alcohol abuse studies
 - GABA_{A5} receptor specificity, 512–516
 - GABA_A benzodiazepine receptor complex, 490–493
 - endogenous ligand sites, 98–99
- Flunitrazepam
 - overview, 637
- Fluorodeoxyglucose (FDG) studies
 - schizophrenia analysis, 257
- Fluoxetine
 - 3,4-methylenedioxymethamphetamine
 - effects on, 616, 627
 - nicotine dependence and withdrawal
 - withdrawal substrate specificity, 542–543
- Fluphenazine
 - adrenergic receptor mechanisms, 427
- Free-radical formation
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity, 624
- Frontal cortex abnormalities
 - glutamate theory of schizophrenia
 - pathological evidence, 292
- Functional candidate genes
 - schizophrenia molecular genetics, 331–332
- Functional imaging studies
 - obsessive-compulsive disorders, 219–220
 - schizophrenia analysis, 257–260
- Fyn tyrosine kinase
 - anxiety neurobiology and, 31
- GABA_A-benzodiazepine receptor complex
 - alcohol abuse studies, 487–501
 - alcohol/modulator commonalities, 487–488
 - CA1/CA3 hippocampus, 511–516
 - efficacy of β CCT/3PBD modulation,
 - GABA_{A1,2,3,5} receptors, 499–501
 - future research issues, 517–518
 - GABA-DA interaction hypothesis, 496–498
 - ligand selectivity with GABA_{A1}
 - subunits, 498–499
 - microinjection studies, 505–506
 - naltrexone antagonist, 507–511
 - novel CNS GABAergic substrates, 498
 - oral administration, β CCT/3PBD
 - anxiety reduction, 510–511
 - vs. naltrexone, 507–511
 - probe applications, 488–493
 - site-specific microinjection, 493–496
 - subunit selectivity vs. intrinsic efficacy, 516–517
 - systemic administration, 492–493, 503–504
 - ventral pallidum, 501–502
- GABAergic neurons
 - alcohol abuse studies
 - in alcohol-preferring rats, 471
 - bed nucleus of stria terminalis system, 474
 - dopamine agonist modification, 496–498
 - GABA_{A5} receptor specificity, 512–516
 - novel substrates, 498
 - ventral pallidum dopaminergic
 - regulation and, 479–480
 - cannabinoid₁ (CB-1) receptors and, 672–673
 - integrated glutamate/dopamine
 - hypotheses of schizophrenia, 375–376
 - psychostimulant abuse, 574–575
 - chronic exposure and sensitization effects, 583–585
 - γ -aminobutyric acid (GABA) receptors
 - and, 576–577
 - novel therapeutic developments, 588

- Gabapentin
 anxiety disorder therapy
 amino acid neurotransmission, 75
 migraine management, 766–767
- GA-BARAP
 GABA_A receptors
 benzodiazepines, 109–110
- "GABA shift" assay
 alcohol abuse studies
 GABA_A benzodiazepine receptor complex
 subunit selectivity *vs.* intrinsic efficacy, 517
- Galantamine
 schizophrenia pharmacotherapy, 393–394
- γ -aminobutyric acid (GABA) receptors
 addictive disorders
 modulator therapy and, 458–459
 anxiety disorder therapy
 amino acid neurotransmission, 72–75
 anxiety neurobiology and, 15–17
 knockout mice deficit studies, 26–30
 cannabinoid receptors and neurotoxicity effects, 673
- GABA_A receptors
 alcohol abuse studies
 novel substrates, 498
 ventral pallidum dopaminergic regulation and, 479–480
 benzodiazepines
 assembly, clustering, and surface expression, 108–110
 binding pocket, 105–108
 brain function diversity, 110–112
 functional diversity, knockout/knockin models, 112–114
 single-cell response modulation, 99–100
 subunit/subtype diversity, 103–104
- GABA_{A1} subunit
 alcohol abuse studies, 498–501
 microinjection techniques, 505–507
 ventral pallidum selectivity, 501–503
- GABA_{A5} subunit
 as alcohol substrate probes, 511–516
 neuroactive steroids, 137–141
- GABA_B receptors
 alcohol abuse studies
 ventral pallidum dopaminergic regulation and, 479–480
- γ -hydroxybutyric acid (GHB)
 mechanisms and, 634–635
 migraine management with anticonvulsants, 766–767
 neuroactive steroids
 enantiomeric selectivity, 141–142
 nicotine dependence and withdrawal
 nicotine reinforcement substrates, 538–540
 NRG1 molecular interaction, 354–355
 psychostimulant abuse and, 576–577
 schizophrenia neurochemistry and, 261–262
- γ -hydroxybutyric acid (GHB)
 addiction risk, 635–636
 mechanism of action, 634–635
 overview, 632–633
 pharmacological effects, 633–634
- "Gate control" theory
 classification of, 713–714
- Geller-Seifter test
 mouse models of anxiety, 8–9
 neurosteroid effects, 145–146
- Gene expression
 psychostimulant abuse
 chronic exposure and sensitization effects, 584–585
- Gene linkage studies
 schizophrenia, 323–324
 future research issues, 355–356
 susceptibility gene identification, 344–351
- Generalized anxiety disorder (GAD)
 buspirone therapy, 71
 pharmacotherapy, 77
 serotonin/noradrenaline reuptake inhibitors, 69
 tricyclic antidepressants, 69–70
- Genetic studies. *See also* Gene linkage studies; Molecular genetics
 addictive disorders vulnerability, 457–458
 anxiety neurobiology, 4–6
 environmental effects and, 34–35
 susceptibility studies, 18–19
 glutamate theory of schizophrenia, 293–294
 obsessive-compulsive disorders, 222–224
 developmental genes, 223–224
 dopamine, 223
 glutamate, 223
 neurotransmitter metabolism, 223
 serotonin, 222–224

- schizophrenia, 262–263
 - animal models, 264
 - dopamine hypothesis, 286–287
 - epidemiology, pathophysiology, and neurobiology, 323
- Gepirone
 - anxiety disorder therapy, 71
- Glia-derived protein
 - GABA_A receptors
 - benzodiazepines, 108–110
- Glucocorticoid receptor (GR)
 - stress-induced behaviors
 - neuroactive steroids, 153–154
- Glucocorticoid receptor (GR) transcription factor
 - anxiety neurobiology and, 31
- Glucocorticoids
 - anxiety neurobiology and, 17
- Glucocortical hormones
 - psychostimulant abuse, 577–578
- Glucose utilization
 - schizophrenia psychosis
 - neural network analysis, 257–258
- Glutamate neurotransmitters
 - anxiety disorder therapy
 - amino acid neurotransmission, 72–75
 - nicotine dependence and withdrawal
 - withdrawal substrates, 542–543
 - obsessive-compulsive disorders, 223
 - pain management and, 717
 - psychostimulant abuse and, 575–576
 - schizophrenia neurochemistry and, 261–262
 - targeted drug development for, 391–392
- Glutamate reuptake inhibitors
 - schizophrenia pharmacotherapy, 391–392
- Glutamatergic system
 - addictive disorders
 - modulator therapy and, 458–459
 - atypical antipsychotics, 427–428
 - nicotine dependence and withdrawal
 - nicotine reinforcement substrates, 538–540
 - psychostimulant abuse, 569–576
 - chronic exposure and sensitization effects, 583–585
 - novel therapeutic developments, 588
 - in schizophrenia, 264
 - molecular genetics
 - functional candidate genes, 331–332
 - pharmacotherapy
 - glutamate reuptake inhibitors, 391–392
- Glutamate theory of schizophrenia
 - dopamine hypothesis and, 295–296
 - genetic evidence for, 293–294
 - history, 289–291
 - imaging evidence for, 292–293
 - neurochemistry of, 373–374
 - pathological evidence for, 291–292
 - pharmacological evidence for, 294–295
- Glutamic acid decarboxylase (GAD)
 - anxiety neurobiology and
 - knockout mice studies, 24–26
 - schizophrenia susceptibility genetics, 350–351
- Glutathione conjugates
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 622
- Glycine
 - schizophrenia pharmacotherapy
 - NMDA targeting with, 391–392
- Glycine transporter 1 (GlyT1)
 - glutamate theory of schizophrenia
 - pharmacological evidence, 294–295
- Glycine transporter inhibitors
 - schizophrenia pharmacotherapy
 - NMDA targeting with, 391–392
- G-protein coupled receptors (GPCRs)
 - corticotropin-releasing factor receptor
 - antagonists
 - ligand binding mechanisms, 192–195
 - delta opioid receptors, 751
 - kappa opioid receptors, 751–752
 - mu opioid receptors and, 748–751
 - regulator of G-protein signaling 4
 - schizophrenia candidate gene, 328
- G-protein-gated inwardly rectifying K⁺ (GIRK) channels
 - anxiety neurobiology and
 - receptor deficits and, 29–30
- G proteins
 - cannabinoid receptors and, 662–663
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 584–585
- GRM3 gene
 - schizophrenia molecular genetics, 331–332
 - susceptibility identification, 349
- Group A β -hemolytic streptococcus (GABHS) infection
 - obsessive-compulsive disorders and, 235

- Guanfacine
 - schizophrenia pharmacotherapy, 393
- Hallucinogens
 - LSD, 637–640
 - addiction, 639–640
 - mechanism of action, 638–639
 - pharmacology, 638
- Haloperidol
 - serotonin receptor 5-HT₆ and, 425–426
- Hamilton depression and anxiety scales
 - corticotropin-releasing factor receptor antagonists
 - anxiety/depression therapeutic potential, 197–198
- Heat exposure
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 730–732
- Hepatitis C
 - opiate addiction comorbidity with, 691–694
- Heroin addiction
 - incidence and prevalence, 692–694
 - neuropeptide pharmacology and, 578–579
 - pharmacotherapy
 - history of, 451–457
 - treatment statistics, 694–696
- High 5-HT_{2A}
 - schizophrenia therapy
 - D₂ affinity *vs.*, 378
- High alcohol drinking (HAD) rats
 - alcohol abuse studies
 - characteristics of, 467
- Hippocampus
 - alcohol abuse studies
 - CA1/CA3 fields
 - GABA_{A5} receptor probes, 511–516
 - anxiety disorders and, 13–15
 - 3,4-methylenedioxymethamphetamine
 - effects on
 - long-term neurochemical effects, 619–620
- Homeobox genes
 - obsessive-compulsive disorders, 223–224
- HTR2A receptor
 - schizophrenia molecular genetics, 331–332
- Human immunodeficiency virus
 - opiate addiction and exposure to, 691–694
- Hydroxyl radicals
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 627
- Hydroxyzine
 - anxiety disorder therapy, 75
- Hyperdopaminergic state
 - schizophrenia dopamine hypothesis and
 - evidence for, 287
- Hyperthermia
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 624–625
- Hypodopaminergia
 - schizophrenia dopamine hypothesis, 288–289
- Hypothalamic-pituitary-adrenal (HPA) axis
 - anxiety neurobiology and, 17
 - corticotropin-releasing factor receptor antagonists, 177–179
 - nicotine dependence and withdrawal
 - nicotine reinforcement substrates, 539–540
 - withdrawal substrate specificity, 543
 - psychostimulant abuse, 577–578
 - chronic exposure and sensitization
 - effects, 580–585
 - stress-induced behaviors
 - neuroactive steroids, 153–159
- Idazozan
 - adrenergic receptor mechanisms, 427
- Iloperidone
 - schizophrenia therapy, 389
- Imaging studies
 - brain regions
 - anxiety disorders and, 13–15
 - glutamate theory of schizophrenia, 292–293
 - schizophrenia
 - dopamine hypothesis, 285–286
- Imidazobenzodiazepines
 - alcohol abuse studies
 - GABA_{A5} receptor specificity, 512–516
- Immediate early genes
 - psychostimulant abuse
 - chronic exposure and sensitization
 - effects, 585
- Immune response
 - 3,4-methylenedioxymethamphetamine
 - effects on, 618
- Immunoglobulin therapy
 - obsessive-compulsive disorders, 235
- Immunomodulatory therapy
 - obsessive-compulsive disorders, 235
- Indole agents
 - serotonin receptor 5-HT_{2A}, 418–421

- Indolealkylamines, 637–640
 - addiction, 639–640
 - mechanism of action, 638–639
 - pharmacology, 638
- Infection
 - obsessive-compulsive disorders and, 235
- Inflammation
 - transient receptor potential V1 (TRPV1) receptor expression, 729
- Integrated glutamate/dopamine hypotheses of schizophrenia
 - basic principles, 295–297
 - pharmacotherapy and, 374–376
- Interleukin-1 β converting enzyme (ICE)
 - 3,4-methylenedioxymethamphetamine effects on, 628
- Interleukin-1 β release
 - 3,4-methylenedioxymethamphetamine effects on, 628
- Intracellular signaling molecules
 - anxiety neurobiology and mouse studies, 19, 22
 - phenotype analysis, 30–33
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 584–585
 - schizophrenia therapy, 394–395
- Intrinsic efficacy
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex
 - subunit selectivity vs., 516–517
- Ionotropic glutamate receptors
 - atypical antipsychotics, 427–429
- J-domain fragments
 - corticotropin-releasing factor receptor antagonists
 - ligand binding mechanisms, 192–195
- Kainate receptors
 - glutamate theory of schizophrenia mRNA binding, 291–292
 - schizophrenia pharmacotherapy drug development for, 392
- Kappa opioid receptors
 - psychostimulant abuse and, 578–579
 - structure and function, 751–752
- Ketamine
 - glutamate theory of schizophrenia history of, 289–290
 - overview, 636
 - pharmacological effects, 636–637
- Ketoconazole
 - psychostimulant abuse, 577–578
- Knockin mice
 - benzodiazepine functional diversity, 112–114
- Knockout mice
 - anxiety neurobiology
 - neuronal messenger alterations, 24–26
 - neurotransmitter receptor/CMAP deficits, 26–30
 - benzodiazepine functional diversity studies, 112–114
 - calcitonin gene-related peptide studies, 760–761
 - transient receptor potential V1 (TRPV1) receptor models, 736
- LAAM (λ - α -Acetyl methadol)
 - addiction pharmacotherapy and, 454–457
 - treatment statistics, 695–696
- Lamotrigine
 - anxiety disorder therapy, 75
 - schizophrenia pharmacotherapy
 - NMDA targeted drug development, 392–393
- Lateral hypothalamus (LH)
 - alcohol abuse studies
 - dopamine neuronal systems and substrates, 481–485
 - D₂ dopaminergic regulation, hypothesized mechanisms, 481–485
- Leptin
 - cannabinoid receptors and, 671–672
- Lesion models
 - in schizophrenia, 265
- Ligand-gated ion channels
 - neuroactive steroids, 137–141
- Light-dark box
 - mouse models of anxiety
 - neurosteroid effects, 146–147
- LIS1 gene
 - DISC1 schizophrenia candidate gene molecular interactions, 351–352
- Lithium
 - anxiety disorder therapy, 75
- Locomotor sensitization
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 581–585

- Lofexidine
 - opiate addiction and, 699
- Long-term therapy
 - selective serotonin reuptake inhibitors, 68–69
- LY206130
 - nicotine dependence and withdrawal withdrawal substrate specificity, 542–543
- LY274600
 - nicotine dependence and withdrawal withdrawal substrate specificity, 542–543
- LY354740 Glu analog
 - nicotine dependence and withdrawal withdrawal substrate specificity, 542–543
- Lysergic acid diethylamide (LSD), 637–640
 - addiction, 639–640
 - mechanism of action, 638–639
 - pharmacology, 638
- M100907 compound
 - schizophrenia therapy
 - serotonin receptor 5-HT_{2A}, 419–421
- Magnetic resonance imaging (MRI)
 - obsessive-compulsive disorders
 - brain imaging studies, 217–218, 220
 - schizophrenia analysis, 256
- Magnetic resonance spectroscopy (MRS)
 - glutamate theory of schizophrenia, 293
 - schizophrenia analysis, 256–257
- MAPK/ERK signaling
 - anxiety neurobiology and, 36–37
- Marijuana
 - endocannabinoid system, 662–665
 - cannabinoid receptors and signaling, 662–663
 - prenatal developmental effects, 664–665
 - reward, tolerance and dependence mechanisms, 675–676
 - synthesis and metabolism, 663–664
 - future research on, 676
 - pharmacology
 - appetite regulation, 670–672
 - cognitive function, 669–670
 - emesis, 673–674
 - endocannabinoid system physiology, 665–666
 - neurotoxicity, 672–673
 - overview, 659–662
 - pain management, 666–669
- Mast cells
 - calcitonin gene-related peptide sites and migraine therapy targeting, 762
- MATRICES program
 - schizophrenia-related cognitive dysfunction, 254–255
- Mazindol
 - psychostimulant abuse therapy, 587
- mCCP serotonin receptor agonist
 - obsessive-compulsive disorders, 234
- Mecamylamine
 - nicotine dependence and withdrawal therapy, 549
- Medial prefrontal cortex (MPFC)
 - anxiety neurobiology
 - brain imaging studies, 15
- Melanocyte-inhibiting factor (MIF) peptides
 - pain management and
- Memantine
 - schizophrenia pharmacotherapy, 392
- Memory deficits
 - cannabinoid receptors and, 669–670
 - 3,4-methylenedioxymethamphetamine and, 630–631
 - schizophrenia
 - animal models, 263
 - schizophrenia and, 254–255
- Mesoaccumbens system
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - GABA_A benzodiazepine receptor complex
 - site-specific microinjection techniques, 494–496
- Mesolimbic pathway
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - psychostimulant abuse
 - dopamine system, 574–575
- Mesopallidal system
 - alcohol abuse studies, 476–480
- Metabolic syndrome
 - antipsychotics and, 254
- Metabolism kinetics
 - endocannabinoid system, 663–664
 - 3,4-methylenedioxymethamphetamine neurotoxicity and, 621–622
 - nicotine dependence and withdrawal, 544–545

- Metabolites
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and mechanisms of, 621–622
- Metabotropic glutamate receptors
 - atypical antipsychotics, 427–429
 - glutamate theory of schizophrenia, 293–294
 - history of, 290–291
 - pathological evidence, 292
 - psychostimulant abuse, 576
 - schizophrenia pharmacotherapy
 - group II receptor targeting, 391–392
- Methadone
 - heroin addiction pharmacotherapy
 - history of, 453–457
 - in "office-based practice," 700
 - opiate addiction therapy
 - buprenorphine comparisons with, 697–698
 - psychostimulant abuse therapy, 585–586
 - treatment statistics, 694–696
- Methamphetamine. *See also* 3,4-Methylenedioxymethamphetamine (MDMA)
 - 3,4-methylenedioxymethamphetamine and
 - behavioral effects, 630–631
 - monoamine neuropharmacology, 571–575
- α -Methylparatyrosine (AMPT)
 - schizophrenia dopamine hypothesis, 372–373
 - schizophrenia therapy
 - D₂ receptor occupancy and effect, 376–378
- Methylation techniques
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 622
- 3,4-Methylenedioxyamphetamine (MDA)
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 621–622
- 3,4-Methylenedioxymethamphetamine (MDMA)
 - behavioral effects, 618–619
 - body temperature effects, 617–618
 - brain biochemistry and function, 631–632
 - monoamine release, 616
 - neuroendocrine and immune responses, 618
 - neurotoxicity, 619–628
 - animal models, 620–621
 - cytokines and microglia, 627–628
 - hyperthermia, 624–625
 - long-term neurochemical change, 619–620
 - metabolite mechanisms, 621–622
 - monoaminergic transporter, 625–627
 - oxidative stress, 623–624
 - neurotoxic lesions
 - behavioral effects, 629–631
 - thermoregulation effects, 629
 - neurotransmitter receptors and transporters, 617
 - overview, 614
 - reinforcing properties, 614–616
 - tryptophan hydroxylase, 616–617
- Methylphenidate
 - chronic exposure and sensitization effects, 580–585
 - monoamine neuropharmacology, 571–575
 - psychostimulant abuse therapy, 587
- Microglia
 - 3,4-methylenedioxymethamphetamine
 - effects on, 627–628
- Microinjection studies
 - alcohol abuse
 - GABA_A benzodiazepine receptor
 - complex modulation, 505–507
- Migraine headaches
 - history and definition, 758–759
 - therapy
 - calcitonin gene-related peptide and, 759–763
 - injection techniques, 763
 - neurovascular model, 759–760
 - receptor antagonist therapeutic efficacy, 763–764
 - synthesis and actions, 760–761
 - trigeminovascular sites, 761–763
 - future trends in, 768–769
 - history of, 758–759
 - migraine diagnostic criteria, 758–759
 - overview, 758
 - pharmacology, 764–768
 - acute therapy, 764–765
 - preventive therapy, 766–768
- Mild mental stress models
 - neurosteroid effects, 151–152
- Mineralocorticoid receptors
 - psychostimulant abuse, 577–578
- Mirrored chamber
 - mouse anxiety models
 - neurosteroid effects, 147–148
- Mirtazapine
 - anxiety disorder therapy, 70

- Missense mutations
 - PRODH gene
 - schizophrenia molecular genetics, 329–330
- Mitogen-activated protein kinase
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 729
- Mitotic inhibitor methylazoxymethanol (MAM)
 - in schizophrenia, 265
- Moclobemide
 - anxiety disorder therapy, 70
- Modafinil
 - psychostimulant abuse therapy, 588
 - schizophrenia therapy, 389
- Modified forced-swim test
 - animal anxiety models
 - neurosteroid effects, 150–151
- Modulation therapy
 - addictive disorders, 458–459
- Molecular genetics
 - corticotropin-releasing factor receptor
 - antagonists
 - ligand binding mechanisms, 191–195
 - GABA_A benzodiazepine receptor complex
 - alcohol abuse studies, 487–501
 - alcohol/modulator commonalities, 487–488
 - CA1/CA3 hippocampus, 511–516
 - efficacy of β CCT/3PBD modulation, GABA_{A1,2,3,5} receptors, 499–501
 - future research issues, 517–518
 - GABA-DA interaction hypothesis, 496–498
 - ligand selectivity with GABA_{A1}
 - subunits, 498–499
 - microinjection studies, 505–506
 - naltrexone antagonist, 507–511
 - novel CNS GABAergic substrates, 498
 - oral administration, β CCT/3PBD
 - anxiety reduction, 510–511
 - vs. naltrexone, 507–511
 - probe applications, 488–493
 - site-specific microinjection, 493–496
 - subunit selectivity vs. intrinsic efficacy, 516–517
 - systemic administration, 492–493, 503–504
 - ventral pallidum, 501–502
 - schizophrenia, 323–325
 - candidate genes, 325–328
 - chromosomal abnormalities, 328–331
 - functional candidate genes, 331–332
 - future research issues, 333
 - gene linkage studies, 323–324
 - neurochemistry and, 261–262
 - positional candidate genes, 325
 - susceptibility genes, 351–355
 - DISC1 gene, 351–352
 - DTNBP1 gene, 352–353
 - function, 332–333
 - NRG1 gene, 353–355
- Molecular path model
 - benzodiazepine activity, 115
- Molecular targeting
 - schizophrenia-related cognitive
 - dysfunction therapy, 254–255
- Monoamine neurotransmitters
 - anxiety disorder anxiolytics, 63–72
 - antidepressants, 64–70
 - antipsychotics, 71–72
 - beta blockers, 71
 - cannabinoid analgesics and, 668–669
 - dopamine hypothesis of schizophrenia, 371–373
 - 3,4-methylenedioxymethamphetamine
 - effects, 616
 - psychostimulant abuse and, 569–575
- Monoamine oxidase inhibitors (MAOIs)
 - anxiety disorder therapy, 70
 - obsessive-compulsive disorders
 - metabolism studies, 223
 - schizophrenia therapy, 394
- Monoaminergic transporter
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 625–627
- Monotherapies
 - obsessive-compulsive disorders
 - controlled trials, 225
- MOR-1 opioid receptor
 - pain management and, 716–717
 - structure and function, 748–751
- Morphine compounds
 - mu opioid receptors and, 748–751
 - obsessive-compulsive disorder therapy, 234
 - peripheral-central interactions, 719–721
 - spinal-supraspinal interactions, 718–720
- Mouse studies
 - anxiety neurobiology
 - anxiety-like behavior, genetically altered mice, 19–24

- neuroticism (N trait), 6–9
- oligogenic anxiety-like conditions, 32–33
- QTL studies, 19
- benzodiazepine functional diversity, 112–114
- MPEP antagonist
 - psychostimulant abuse and, 576
- Multipoint linkage analysis
 - anxiety neurobiology, 4
- Mu opioid receptors
 - obsessive-compulsive disorders, 234
 - psychostimulant abuse and, 578–579
 - structure and function, 748–751
- Muscarinic receptor agonists
 - atypical antipsychotics and cholinergic agents, 429–430
 - schizophrenia therapy, 393–394
- Muscimol
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex manipulation, 494–496
- Naloxone
 - nicotine dependence and withdrawal, 540
 - opiate addiction therapy
 - regulatory studies, 696–697
- Naltrexone
 - alcohol abuse studies
 - oral administration of β CCT/3PBC vs., 507–511
 - nicotine dependence and withdrawal, 540
 - pharmacotherapy, 550–551
 - opiate addiction therapy, 698–699
 - psychostimulant abuse therapy, 585–586
- NAN-190 antagonists
 - nicotine dependence and withdrawal
 - withdrawal substrate specificity, 542–543
- N-back task analysis
 - schizophrenia cognitive dysfunction and, 259
- NDMC metabolite
 - cholinergic mechanisms and, 429–430
- N-domain fragments
 - corticotropin-releasing factor receptor antagonists
 - ligand binding mechanisms, 192–195
- Negative affect
 - in schizophrenia, 255–256
 - neural network studies, 258–259
- NEO-five factor inventory (NEO-FFI)
 - anxiety neurobiology
 - genetic susceptibility studies, 18–19
- NEO personality inventory
 - anxiety neurobiology, 5–6
 - human personality traits, 9–12
- Neospinothalamic pain pathways
 - classification of, 711–714
- Nerve-growth factor (NGF)
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 729
- Neural networks
 - glutamate theory of schizophrenia
 - history of, 290–291
 - schizophrenia
 - cognitive dysfunction and, 259
 - future research issues, 266
 - negative affect and, 255–256, 258–259
 - psychosis and, 257–258
- Neuregulin I (NRG1)
 - schizophrenia candidate gene, 326–327
 - ErbB3 receptor, 328
 - functional implications, 332–333
 - molecular interactions, 352–353
 - susceptibility identification, 350
- Neuroactive steroids
 - anxiety disorders
 - alcohol effects, 157–159
 - animal models, 142–152
 - behavioral effects, 142
 - brain and peripheral sources, 135–137
 - chemistry and pharmacology, 135–142
 - enantiomeric selectivity, 141–142
 - GABA_A receptors and ligand-gated ion channels, 137–141
 - HPA axis function, 154–156
 - overview, 134–135
 - stress-induced behaviors
 - drug abuse relapse, 159–160
 - HPA axis, 153–154
 - overview, 152–153
- Neuroanatomical controls
 - alcohol abuse studies in alcohol-preferring rats, 469–470
- Neurobiology
 - obsessive-compulsive disorders, 216–224
 - brain imaging studies, 217–222
 - functional imaging studies, 219–220
 - magnetic resonance spectroscopy, 220

- Neurochemistry
 - 3,4-Methylenedioxymethamphetamine (MDMA) neurotoxicity
 - long-term changes in, 619–620
 - schizophrenia, 260–262
 - hypotheses, 371–376
 - dopamine hypothesis, 371–373
 - glutamate receptor hypothesis, 373–374
 - integrated dopamine/glutamate hypotheses, 374–376
- Neurodevelopmental animal model
 - schizophrenia, 265
- Neuroendocrine systems
 - 3,4-methylenedioxymethamphetamine effects on, 618
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 580–585
- Neurogenesis
 - atypical antipsychotics and, 430
 - schizophrenia
 - disruption of, 265
- Neurogenic inflammation
 - vanilloid receptors and, 728–729
- Neuroimaging studies
 - 3,4-methylenedioxymethamphetamine neurotoxicity
 - brain biochemical and functional changes, 631–632
 - obsessive-compulsive disorders, 219–220
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 582–585
 - monoamine neuropharmacology, 573–575
- Neurokinin 3 receptors
 - antipsychotic mechanisms with, 430
- Neurokinin antagonists
 - schizophrenia therapy, 391
- Neuronal cell adhesion molecules (NCAM)
 - anxiety neurobiology, 30
- Neuronal pathways
 - anxiety neurobiology and, 15–18
 - knockout mice studies, 24–26
 - mouse studies, 19–20
 - pain management
 - anatomical drug interactions, 718–720
 - descending modulatory pathways, 712–714
 - neospinothalamic/paleospinothalamic pathways, 711–712
 - overview, 709–710
- Neuronal systems and substrates
 - calcitonin gene-related peptide sites and migraine therapy targeting, 762
 - dopaminergic receptors
 - alcohol abuse studies, 471–486
 - bed nucleus of stria terminalis, 473–475
 - dopaminergic regulation, early research, 472–473
 - extracellular dopamine, 485–486
 - future research issues, 517–518
 - GABAergic interactions with, 486
 - lateral hypothalamus, 481–485
 - D₂ dopaminergic regulation, hypothesized mechanisms, 481–485
 - ventral pallidum, 476–480
 - dopaminergic regulation hypothesized mechanisms, 478–480
 - nicotine dependence and withdrawal
 - nicotine reinforcement substrates, 537–540
 - withdrawal neurosubstrates, 541–543
- Neuropeptide FF
 - pain management and, 716–717
- Neuropeptides
 - anxiety neurobiology, 16–17
 - psychostimulant abuse and, 578–579
- Neuropeptide Y (NPY)
 - alcohol abuse studies
 - in alcohol-preferring rats, 471
 - anxiety neurobiology and, 17
 - knockout mice studies, 25–26
- Neuropharmacology
 - obsessive-compulsive disorders, 225–235
 - antipsychotic agents, 229–234
 - augmenting agents, 225, 229–234
 - brain imaging studies, 220–222
 - current trials, 234
 - monotherapy trials, 225
 - serotonin uptake inhibitor efficacy, 226–228
 - schizophrenia dopamine hypothesis, 287
- Neuroprotective agents
 - 3,4-methylenedioxymethamphetamine neurotoxicity and, 625
- Neuroreceptor imaging
 - schizophrenia, 259–260

- Neurosteroids
 - schizophrenia therapy, 394
- Neurosurgery
 - obsessive-compulsive disorders, 235–237
- Neurotensin agonist/antagonist
 - psychostimulant abuse and, 579
 - schizophrenia therapy, 390
- Neuroticism (N trait)
 - anxiety neurobiology, 5–6
 - mouse behavior, 6–9
- Neurotoxicity
 - cannabinoid receptors and, 672–673
 - 3,4-Methylenedioxymethamphetamine (MDMA), 619–628
 - animal models, 620–621
 - cytokines and microglia, 627–628
 - hyperthermia, 624–625
 - long-term neurochemical change, 619–620
 - metabolite mechanisms, 621–622
 - monoaminergic transporter, 625–627
 - oxidative stress, 623–624
- Neurotransmitters. *See also* specific
 - Neurotransmitters
 - addictive disorders
 - modulator therapy and, 458–459
 - anxiety neurobiology
 - knockout mice studies, 24–26
 - anxiety neurobiology and, 15–18
 - endocannabinoid system, 663
 - 3,4-methylenedioxymethamphetamine
 - effects on
 - receptor/transporter effects, 617
 - obsessive-compulsive disorders
 - metabolism studies, 223
 - pain neuropharmacology, 714–717
 - drug action localization, 718
 - psychostimulant abuse
 - chronic exposure and sensitization
 - effects, 580–581, 583–585
- Neurovascular model
 - migraine therapy, calcitonin gene-related peptide, 759–760
- NF- κ B transcription factor family
 - anxiety neurobiology and, 31
- Nicotine
 - basic properties, 535–536
 - patches
 - obsessive-compulsive disorder therapy, 234
 - pharmacology, 536–545
 - absorption pharmacokinetics, 543–544
 - dependence and withdrawal therapy
 - bupropion, 549–550
 - cannabinoid antagonists, 550
 - nicotinic antagonists, 548–549
 - nicotinic partial agonists, 548
 - nonnicotinic agents, 549–551
 - opioid antagonists, 550–551
 - overview, 545–546
 - public health policy and, 551–552
 - replacement medications, 546–548
 - tricyclic antidepressants, 550
 - distribution pharmacokinetics, 544
 - metabolism and elimination
 - pharmacokinetics, 544–545
 - nicotine reinforcement neurosubstrates, 537–540
 - nicotinic acetylcholine receptors, 537
 - functional adaptations, 540–541
 - withdrawal neurosubstrates, 541–543
- Nicotine gum
 - nicotine dependence and withdrawal therapy, 547–548
- Nicotine-*N*-oxide
 - nicotine dependence and withdrawal
 - metabolism and elimination
 - pharmacokinetics, 544–545
- Nicotinic acetylcholine receptors (nAChRs)
 - nicotine dependence and withdrawal
 - classification and function, 537
 - functional adaptations mechanisms, 540–541
 - nicotine reinforcement substrates, 538–540
 - nicotinic partial agonist therapy, 548
 - nicotinic partial antagonist therapy, 548–549
 - overview, 536–537
 - withdrawal substrates, 542–543
- NRG1 molecular interaction, 354–355
- schizophrenia pharmacotherapy
 - cholinergic agents, 393–394
- Nicotinic agonists
 - anxiety neurobiology and
 - receptor deficits and, 29–30
- Nicotinic antagonists
 - nicotine dependence and withdrawal therapy, 548
- Nicotinic partial agonists
 - nicotine dependence and withdrawal therapy, 548

- Nitric oxide synthase (NOS) inhibitor
3,4-methylenedioxymethamphetamine
neurotoxicity, 623–624
- Nitrogen ohne radikal (NOR) metabolites
benzodiazepine receptor ligand
metabolism, 102
- NMDA (*N*-methyl-D-aspartic acid)
receptors
addictive disorder pharmacotherapy
methadone interactions, 456–457
consolidated glutamate/dopamine
hypotheses, 295–296
glutamate theory of schizophrenia
history of, 289–290
imaging studies, 293
neurochemistry of, 373–374
pathological evidence, 291–292
pharmacological evidence, 294–295
ketamine effects on, 636–637
obsessive-compulsive disorders
glutamate genetic studies, 223
pain management and, 717
schizophrenia pharmacotherapy
first- and second-generation
antipsychotics, 381
targeted drug development for, 391–392
schizophrenia psychosis, 257–258
- Nociception
defined, 710
drug action localization, 718
pain pathways and, 711–712
transient receptor potential V2 (TRPV2),
736–737
- Nonopiate analgesics
migraine management, 765
- Nonpeptide ligands
corticotropin-releasing factor receptor
antagonists, 195–196
- Nonpharmacological therapy
obsessive-compulsive disorders and,
235–237
- Nonsteroidal anti-inflammatory drugs
migraine management, 765, 768
- Noradrenergic systems
psychostimulant abuse
chronic exposure and sensitization
effects, 584
schizophrenia pharmacotherapy, 393
- Norepinephrine (NE)
alcohol abuse studies
dopaminergic receptor systems and
substrates, 472–473
anxiety neurobiology, 15–17
3,4-methylenedioxymethamphetamine
effects on, 616
receptor/transporter effects, 617
psychostimulant abuse
chronic exposure and sensitization
effects, 584
neuropharmacology, 570–575
schizophrenia pharmacotherapy
noradrenergic agent development, 393
- Norepinephrine transporter (NET)
anxiety neurobiology and
knockout mice studies, 24–26
- Nucleus accumbens (NAcc)
alcohol abuse studies
in alcohol-preferring rats, 470–471
dopaminergic receptor systems and
substrates, 471–472
GABA_A benzodiazepine receptor
complex manipulation
site-specific microinjection studies,
493–496
3,4-methylenedioxymethamphetamine
(MDMA)
reinforcing properties, 614–616
- NUDEL gene
DISC1 schizophrenia candidate gene
molecular interactions, 351–352
- Nur transcription factors
schizophrenia therapy
dopamine neurotransmission and,
422
- Obsessive-compulsive disorders (OCD)
animal models, 224
brain regions related to, 13–15
clinical psychopharmacology, 225–235
antipsychotic agents, 229–234
augmenting agents, 225, 229–234
current trials, 234
monotherapy trials, 225
serotonin uptake inhibitor efficacy,
226–228
deep brain stimulation, 236–237
defined, 216
diagnostic criteria, 10–12, 216
genetic studies, 222–224
developmental genes, 223–224
dopamine, 223
glutamate, 223
neurotransmitter metabolism, 223
serotonin, 222–224

- immunomodulatory treatments, 235
- neurobiology, 216–224
 - brain imaging studies, 217–222
 - functional imaging studies, 219–220
 - magnetic resonance spectroscopy, 220
- neuropharmacology
 - brain imaging studies, 220–222
- neurosurgery, 235–237
- nonpharmacological experimental treatments, 235–237
- pharmacotherapy, 77–78
- serotonin/noradrenaline reuptake inhibitors, 69
- summary of therapeutic advances, 237
- symptom induction, 234
- transcranial magnetic stimulation, 236
- tricyclic antidepressants, 69–70
- Ocapridone
 - schizophrenia therapy, 389
- 8-OH-DPAT
 - nicotine dependence and withdrawal
 - withdrawal substrate specificity, 542–543
 - serotonin receptors 5-HT_{2A}-5-HT_{2C} interactions, 422–424
- Olanzapine
 - D₂ receptor blockade, 414–415
 - neurogenesis and, 430
 - schizophrenia therapy
 - brain-derived neurotrophic factor and, 421–422
 - clinical profile, 383–387
 - D₂ receptor occupancy and effect, 376–378
 - safety and tolerability, 388–389
 - serotonin receptor 5-HT_{2A}, 420–421
- Oligogenic anxiety-like conditions
 - anxiety neurobiology
 - mouse studies, 32–33
- Open-field activity
 - mouse anxiety models
 - neurosteroid effects, 148–149
- Opiates
 - addiction and
 - buprenorphine studies, 696–698
 - drug discovery survey, 699–700
 - epidemiology, 691–694
 - future research issues, 698–699
 - treatment statistics, 694–696
 - pain management
 - anatomical interactions, 718–720
- Opioid agonists/antagonists
 - migraine management, 765
 - nicotine dependence and withdrawal therapy, 550–551
- Opioid receptor system
 - addictive disorders
 - reward therapy and, 458–459
 - alcohol abuse studies
 - oral administration of β CCT/3PBC vs. agonists, 507–511
 - delta receptors, 751
 - dimerization, 752
 - endogenous opioids, 746–748
 - future research on, 752
 - kappa receptors, 751–752
 - mu receptors, 748–751
 - nicotine dependence and withdrawal, 540
 - withdrawal substrate specificity, 543
 - orphanin FQ/nociceptin and receptor, 752
 - overview, 745–746
 - pain management and, 716–717
 - targeting mechanisms, 718
 - psychostimulant abuse and, 578–579
 - chronic exposure and sensitization effects, 584
 - short-acting
 - addiction pharmacotherapy and, 453–457
- Orbitofrontal-dorsomedial thalamic loop
 - obsessive-compulsive disorders
 - neuropharmacology, 221–222
- Orphanin FQ/nociceptin (OFQ/N) receptor
 - anxiety neurobiology and
 - knockout mice studies, 25–26
 - structure and function, 748, 752
- Oxidative stress
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 623–624
- Oxycodone
 - abuse of
 - incidence and prevalence, 692–694
- Pain perception and management
 - calcitonin gene-related peptide for, 768–769
 - marijuana and, 666–668
 - neuronal pathways
 - descending modulatory pathways, 712–714
 - neospinothalamic/paleospinothalamic pathways, 711–712
 - overview, 709–710

- neuropharmacology, 714–717
 - anatomically-based interactions, 718–721
 - drug targeting mechanisms, 718
- transient receptor potential V1 (TRPV1) receptors
 - antagonists, 733
 - capsaicin, protons, and heat, 730–732
 - chemical activators, 732–733
 - cloning of, 728–729
 - desensitization, 735–736
 - expression, 729
 - knockout mouse models, 736
 - nociception channels, 736–737
 - sensitization, 733–735
- Paleospinothalamic pain pathways
 - classification of, 711–712
- Paliperidone
 - schizophrenia therapy, 389
- Panic disorders
 - anticonvulsant therapy, 74–75
 - anxiety disorder therapy, 78
 - brain regions related to, 13–15
 - diagnostic criteria, 10–12
 - neuroactive steroids, 155
- Peptide ligands
 - corticotropin-releasing factor receptor antagonists
 - basic properties, 180–181
 - CRF₁/CRF₂ binding mechanisms, 190–195
 - CRF₁/CRF₂ pharmacology, 185–195
 - CRF₁/CRF₂ receptor pharmacology, 185–195
 - structure and function, 179–185
 - subtypes and distribution, 181–185
- Periaqueductal gray (PAG) stimulation
 - cannabinoid analgesics and, 667–668
- Peripheral benzodiazepine receptors (PBRs)
 - stress-induced behavior
 - neuroactive steroids, 155–156
- Personality traits
 - continuous expression
 - anxiety neurobiology, 9–12
- Pharmacophore models
 - benzodiazepine activity, 116
- Pharmacotherapy
 - addictive disorders
 - history of, 451–457
 - reward modulation/countermodulation and, 458–459
 - risk factors for addiction development, 457–458
- anxiety disorders
 - anxiolytic drugs, 62–75
 - amino acid neurotransmission, 72–75
 - anticonvulsants, 74–75
 - antidepressants, 64–70
 - antihistamines, 75
 - antipsychotics, 71–72
 - benzodiazepines, 73–74
 - beta-blockers, 71
 - lithium, 75
 - monoamine neurotransmission, 63–72
 - serotonin receptor agonists, 70–71
 - depressive disorders, 80
 - future research issues, 81
 - generalized anxiety disorder, 77
 - obsessive-compulsive disorder, 77–78
 - overview, 60
 - panic disorder/agoraphobia, 78
 - phobias, 78, 80
 - posttraumatic stress disorder, 79
 - social anxiety disorder, 79–80
 - treatments chart, 76
- obsessive-compulsive disorders
 - overview, 216
- schizophrenia
 - antipsychotic drug profiles
 - first-generation (conventional) agents, 383
 - safety and tolerability, 387–388
 - second-generation (atypical) agents, 383–387
 - antipsychotic mechanisms of action, 376–382
 - D₁ receptors, 380–381
 - D₂/D₃ and D₄ antagonism and regional specificity, 380
 - D₂ occupancy thresholds and rapid dissociation, 378–380
 - D₂ receptor occupancy, 376–378
 - dopamine release, 381
 - NMDA receptor function, 381
 - synthesis reactions, 381–382
 - current developments and future directions, 388–394
 - cannabinoid hypothesis, 394
 - cholinergic agents, 393–394
 - D₁ agonists and antagonists, 390
 - D₃ antagonists, 390
 - D₄ antagonists, 390

- dopamine system targeting, 389
- glutamate system targeting, 391–393
- neurokinin antagonists, 391
- neurosteroids, 394
- neurotensin agonist/antagonist, 390
- noradrenergic agents, 393
- future research issues, 394–395
- high 5-HT_{2A} vs. D₂ affinity, 378
- neurochemical hypotheses, 371–376
- overview, 370–371
- Phencyclidine. *See also* Ketamine
 - glutamate theory of schizophrenia
 - history of, 289–290
 - neurochemistry of, 374
 - ketamine derivative, 636–637
 - schizophrenia neurochemistry and, 260–262
- Phenothiazines
 - schizophrenia therapy
 - clinical profiles, 383
- Phenotype analysis
 - obsessive-compulsive disorders, 237
 - schizophrenia, 262–263
 - genetic epidemiology, 322–323
- Phenylalkylamines, 637–640
 - addiction, 639–640
 - mechanism of action, 638–639
 - pharmacology, 638
- α -Phenyl-*N*-*tert*-butyl nitron (PBN)
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity
 - oxidative stress, 623–624
- Phobias
 - brain regions related to, 13–15
 - pharmacotherapy, 79–80
- Phosphoinositol 3-kinase-AKT signaling
 - pathway
 - dystrobrevin binding protein 1 (DTNBP1)
 - molecular interactions, 353
- Phosphoinositol-4,5-bisphosphate (PIP₂)
 - transient receptor potential V1 (TRPV1)
 - receptor sensitization, 734–735
- Phospholipase C
 - transient receptor potential V1 (TRPV1)
 - receptor sensitization, 734–735
- Phosphorus MRS
 - schizophrenia analysis, 257
- Picrotoxin
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex, 487–488
- GABAergic modification of dopamine
 - agonists, 496–497
- p-MPPI receptor antagonist
 - nicotine dependence and withdrawal
 - withdrawal substrate specificity, 542–543
- Positional candidate genes
 - schizophrenia genetics, 325
- Positional cloning
 - schizophrenia genetics, 324
- Positron emission tomography (PET)
 - obsessive-compulsive disorders, 219–220
 - schizophrenia
 - dopamine hypothesis, 286
 - schizophrenia analysis, 257
- Postmortem studies
 - schizophrenia, 260–262
 - dopamine hypothesis and, 285
 - future research issues, 355–356
 - overview, 343–344
 - susceptibility gene identification, 344–351
 - susceptibility gene interactions, 351–355
- Postpartum depression
 - neuroactive steroids, 156–157
- Posttraumatic stress disorder (PTSD)
 - anticonvulsant therapy, 74–75
 - brain regions related to, 13–15
 - diagnostic criteria, 10–12
 - mirtazapine therapy, 70
 - neuroactive steroids, 154–155
 - pharmacotherapy, 79
- PPP3CC gene
 - schizophrenia molecular genetics, 328
- P rat line. *See* Alcohol-preferring rats
- Predictive validity
 - animal anxiety-like behavior and, 12
- "Preemptive analgesia"
 - pain management and, 717
- Prefrontal cortex (PFC)
 - integrated glutamate/dopamine
 - hypotheses of schizophrenia, 374–376
- Pregnancy
 - marijuana effects in, 664–665
 - 3,4-methylenedioxymethamphetamine and, 630–631
- Pregnenolone sulfate (PREGS)
 - in brain, 137
- Prenatal development
 - marijuana effects on, 664–665

- Prescription drugs. *See also* Opiates
 abuse of
 incidence and prevalence, 692–694
 Preventive therapy
 migraine management, 766–768
 PRODH gene
 schizophrenia molecular genetics, 329–330
 Prodromal period
 schizophrenia therapy
 second-generation antipsychotics,
 386–387
 Progesterone levels
 depression
 neuroactive steroids, 156–157
 Pro-opiomelanocortin peptide group
 nicotine dependence and withdrawal, 540
 3-Propoxy- β -carboline hydrochloride
 (3BPC)
 alcohol abuse studies
 anxiety reduction with, 510–511
 GABA_{A1,2,3,5} receptor subunit
 modulation, 499–501
 GABA_{A1} receptor subunit selectivity,
 498–499
 microinjection techniques, 505–507
 oral administration, 507–511
 systemic administration, 503–504
 Protein kinase A
 transient receptor potential V1 (TRPV1)
 receptor sensitization, 733–735
 Protein kinase C
 transient receptor potential V1 (TRPV1)
 receptor sensitization, 733–735
 Protein kinase C _{γ}
 anxiety neurobiology and, 31
 Protons
 transient receptor potential V1 (TRPV1)
 receptor expression, 730–732
 Psilocybin
 schizophrenia therapy
 serotonin receptor 5-HT_{2A}, 420–421
 Psychoeducation
 anxiety management with, 60–61
 Psychological traits
 anxiety neurobiology
 continuous expression of normal
 personality, 9–10
 genetic basis of, 4–6
 mouse behavior extrapolation studies,
 6–9
 Psychosis
 in schizophrenia, 253–254
 animal models, 264–265
 neural network studies, 257–258
 second-generation antipsychotics and,
 386–387
 Psychostimulants
 abuse-related neuropharmacology,
 569–579
 γ -aminobutyric acid, 576–577
 glutamate, 575–576
 hypothalamic-pituitary-adrenal axis,
 577–578
 monoamines, 570–575
 neuropeptides, 578–579
 addiction therapy development,
 585–586
 chronic exposure-related neurobiology,
 579–589
 neurotransmitter/neuroendocrine
 systems, 580–584
 signal transduction mechanisms and
 gene expression, 584–585
 future research issues, 588–589
 therapeutic applications of, 567–569
 Public health policy
 nicotine dependency and withdrawal
 therapy, 551–552
 Pyrazolopyrimidine
 corticotropin-releasing factor receptor
 antagonists
 anxiety/depression therapeutic
 potential, 197–198
 Pyrazoloquinoline
 alcohol abuse studies
 GABA_A benzodiazepine receptor
 complex, 490–493
 Quantitative behavioral genetics
 anxiety neurobiology, 6
 Quantitative trait locus (QTL) analysis
 anxiety neurobiology, 4
 emotionality studies in mice, 8–9
 mice studies, 19
 oligogenic anxiety-like conditions,
 32–33
 Quetiapine
 schizophrenia therapy
 clinical profile, 383–387
 safety and tolerability, 389
 Quinelorane
 alcohol abuse studies
 dopaminergic receptor systems and
 substrates, 472–473

- Quinpirole
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
- Raclopride
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - chronic exposure and sensitization effects, 582–585
- ¹¹C-Raclopride
 - striatal D₂ receptor blockade, 414–415
- Radiolabeled peptides
 - corticotropin-releasing factor receptor antagonists
 - peptide ligand pharmacology, 187–195
- Rapid dissociation
 - schizophrenia pharmacotherapy
 - D₂ receptor occupancy thresholds, 379–380
- Receptor activity modifying protein 1 (RAMP1)
 - calcitonin gene-related peptide sites and migraine therapy targeting, 762
- Receptor density assessment
 - schizophrenia
 - dopamine hypothesis and, 285
 - schizophrenia neuroreceptor imaging, 259–260
- Receptor ligand pharmacology
 - benzodiazepines, 97–102
 - endogenous site, 98–99
 - metabolism functions, 101–102
 - single-cell GABA response modulation, 99–100
 - therapeutic action, 97–98
 - tolerance and dependence
 - characteristics, 100–101
- Regional cerebral blood flow (rCBF)
 - schizophrenia, 257–259
- Regional specificity
 - schizophrenia therapy
 - D₂/D₃ and D₄ receptor antagonism and, 380
- "Region-specific" neuroanatomical controls
 - alcohol abuse studies in alcohol-preferring rats, 469–470
- Regulator of G-protein signaling 4 (RGS4)
 - schizophrenia candidate gene, 328
 - susceptibility identification, 351
- Reinforcing mechanisms
 - alcohol abuse studies in alcohol-preferring rats, 469
 - 3,4-methylenedioxymethamphetamine (MDMA), 614–616
- Resiniferatoxin (RTX)
 - cloning of, 727–729
 - transient receptor potential V1 (TRPV1) receptor expression, 733
- Respiratory system
 - γ-hydroxybutyric acid (GHB) effects, 634
- Reuptake inhibitors
 - psychostimulant abuse
 - monoamine neuropharmacology, 571–575
- Reversible inhibitor of monoamine oxidase A (RIMA)
 - anxiety disorder therapy, 70
- Reward effects
 - addictive disorders, 458–459
 - endocannabinoids and, 675–676
 - 3,4-methylenedioxymethamphetamine (MDMA), 614–616
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 581–585
- Rhodopsin
 - corticotropin-releasing factor receptor antagonists
 - ligand binding mechanisms, 191–195
- Riluzole
 - obsessive-compulsive disorder therapy, 234
- Rimonabant
 - appetite regulation, 670–672
 - cannabinoid receptors and, 661–662
 - cognition and, 669–670
 - endocannabinoid physiology and, 665–675
 - nicotine dependence and withdrawal therapy, 550
 - opiate dependence therapy and, 699
 - pain management and, 667–668
 - reward, tolerance, and dependence, 675–676
- Risperidone
 - neurogenesis and, 430
 - schizophrenia therapy
 - clinical profile, 383–387
 - safety and tolerability, 389
 - serotonin receptor 5-HT_{2A}, 418–421

- Ritanserin
3,4-methylenedioxymethamphetamine
effects on, 616
- RO19-4603 inverse agonist
alcohol abuse studies
GABA_A benzodiazepine receptor
complex, 488–493
site-specific microinjection
techniques, 494–496
systemic administration studies,
492–493
- Rohypnol. *See* Flunitrazepam
- ROI15-4513 inverse agonist
alcohol abuse studies
GABA_A benzodiazepine receptor
complex
alcohol-modulator commonalities,
487–488
- RU34000 imidazopyrimidine inverse agonist
alcohol abuse studies
GABA_A benzodiazepine receptor
complex, 490–493
site-specific microinjection
techniques, 496
- RY 023 inverse agonist
alcohol abuse studies
GABA_{A5} receptor specificity,
512–516
- Safety
schizophrenia therapy
second-generation antipsychotics,
387–388
- Samson test procedure
alcohol abuse studies in alcohol-preferring
rats, 468–470
- SB-271046
serotonin receptor 5-HT₆ and,
425–426
- SB-399885
serotonin receptor 5-HT₆ and, 426
- SCH 23390 compound
alcohol abuse studies
bed nucleus of stria terminalis system,
475
lateral hypothalamus, effects on,
481–485
ventral pallidum receptor blockade,
477–480
- Schizophrenia
animal models, 263–265
biological mechanisms, 256–263
brain imaging studies, 256–260
characteristics of, 252
clinical phenomenology and treatment,
252–256
cognitive dysfunction, 254–255
dopamine hypothesis
cortical vs. striatal dopamine, 287–289
genetic evidence, 286–287
history, 284–285
imaging evidence, 285–286
pathological evidence, 285
pharmacological evidence, 287
pharmacotherapy, 371–373
evolution of theories on, 283–284
future research issues, 265–266
genetic epidemiology, 321–323
phenotype definition, 322–323
genetics and phenotypes, 262–263
glutamate theory of
genetic evidence for, 293–294
history, 289–291
imaging evidence for, 292–293
pathological evidence for, 291–292
pharmacological evidence for,
294–295
pharmacotherapy, 373–374
integrated glutamate/dopamine
hypotheses, 295–297
pharmacotherapy, 374–376
molecular genetics, 323–325
candidate genes, 325–328
chromosomal abnormalities, 328–331
functional candidate genes, 331–332
future research issues, 333
susceptibility gene function, 332–333
negative affect, 255–256
neural network studies and, 255–259
neurochemistry, 260–262
neurogenesis in, 430
neuroreceptor imaging, 259–260
pharmacotherapy
antipsychotic drug profiles
first-generation (conventional)
agents, 383
safety and tolerability, 387–388
second-generation (atypical) agents,
383–387
antipsychotic mechanisms of action,
376–382
D₁ receptors, 380–381
D₂/D₃ and D₄ antagonism and
regional specificity, 380

- D₂ occupancy thresholds and rapid dissociation, 378–380
- D₂ receptor occupancy, 376–378
- dopamine release, 381
- NMDA receptor function, 381
- synthesis reactions, 381–382
- current developments and future directions, 388–394
- cannabinoid hypothesis, 394
- cholinergic agents, 393–394
- D₁ agonists and antagonists, 390
- D₃ antagonists, 390
- D₄ antagonists, 390
- dopamine system targeting, 389
- glutamate system targeting, 391–393
- neurokinin antagonists, 391
- neurosteroids, 394
- neurotensin agonist/antagonist, 390
- noradrenergic agents, 393
- future research issues, 394–395
- high 5-HT_{2A} vs. D₂ affinity, 378
- neurochemical hypotheses, 371–376
- overview, 370–371
- postmortem studies, 260–262
- dopamine hypothesis and, 285
- future research issues, 355–356
- overview, 343–344
- susceptibility gene identification, 344–351
- susceptibility gene interactions, 351–355
- prevalence, 252–253
- psychosis and, 253–254
- symptom classification, 253
- Second-generation antipsychotics (SGAs)
- schizophrenia therapy
- clinical profile, 383–387
- D₂/D₃ and D₄ receptor antagonism and regional specificity, 380
- D₂ receptor occupancy thresholds and rapid dissociation, 378–380
- history of, 370–371
- NMDA receptor antagonists, 381
- safety and tolerability, 387–388
- Selective serotonin reuptake inhibitors (SSRIs)
- anxiety disorder therapy, 66–69
- obsessive-compulsive disorders
- controlled trials, 225
- panic disorder therapy, 78
- posttraumatic stress disorder therapy, 79
- social anxiety disorder therapy, 79–80
- Selegiline
- schizophrenia therapy, 394
- Sensitivity
- psychostimulant abuse and reduction of neurobiology of, 579–585
- transient receptor potential V1 (TRPV1) receptor expression, 733–735
- Sensory neurons
- pain pathways, 711–712
- Separation-induced ultrasonic vocalizations
- animal anxiety models
- neurosteroid effects, 150
- Sequence homology
- corticotropin-releasing factor receptor antagonists
- ligand binding mechanisms, 191–195
- Serotonin (5-HT)
- antipsychotic drugs and release of, 426
- anxiety disorder anxiolytics
- monoamine neurotransmission, 63–72
- anxiety neurobiology, 4
- genetic susceptibility studies, 18–19
- neurotransmission mechanisms, 15–17
- 3,4-methylenedioxymethamphetamine
- effects on, 616
- long-term neurochemical effects, 619–620
- oxidative stress, 623–624
- receptor/transporter effects, 617
- nicotine dependence and withdrawal
- withdrawal substrates, 542–543
- obsessive-compulsive disorders
- genetic studies, 222–224
- psychostimulant abuse
- neuropharmacology, 570–575
- Serotonin and noradrenaline reuptake inhibitors (SNRI)
- anxiety disorder therapy, 69
- Serotonin hypothesis
- obsessive-compulsive disorders
- neuropharmacology, 221–222
- Serotonin receptor 5-HT/D₂ hypothesis
- atypical antipsychotics, 417–418
- serotonin receptor 5-HT_{2A}, 419–421
- Serotonin receptors
- atypical antipsychotics
- adrenergic mechanisms, 426–427
- serotonin receptor 5-HT_{1A}-5-HT_{2A} interactions, 421–425
- serotonin receptor 5-HT_{2A}, 418–421
- cortical dopamine efflux and cognitive function, 421–422
- extrapyramidal function, 422

- serotonin receptor 5-HT_{2A}-5-HT_{2C}
 - receptor interactions, 422–424
- serotonin receptor 5-HT/D₂ hypothesis, 417–418
- schizophrenia neurochemistry and, 261–262
- schizophrenia pharmacotherapy
 - serotonin receptor 5-HT_{2A}
 - D₂ antagonism and, 381
- serotonin 5-HT_{1A}
 - nicotine dependence and withdrawal, 542–543
- serotonin 5-HT_{2C} receptors
 - psychostimulant abuse, 574–575
- serotonin receptor 5-HT_{1A}
 - anxiety disorder therapy, 70–71
 - anxiety neurobiology and
 - knockout mice deficit studies, 27–30
- serotonin receptor 5-HT_{1B}
 - anxiety neurobiology and
 - knockout mice deficit studies, 28–30
- serotonin receptor 5-HT_{2A}
 - cortical dopamine efflux and cognitive function, 421–422
 - extrapyramidal function, 422
 - LSD downregulation of, 638–640
 - schizophrenia pharmacotherapy
 - D₂ antagonism and, 381
- serotonin receptor 5-HT₆ interactions
 - atypical antipsychotics, 425–426
- triptan therapy for migraines, 764–765
- Serotonin reuptake inhibitors (SRIs)
 - obsessive-compulsive disorders
 - brain neuropharmacology, 221–222
 - neuropharmacology, 226–228
- Serotonin transporter (5-HTT)
 - anxiety neurobiology
 - environmental effects and, 34–35
 - anxiety neurobiology and
 - knockout mice studies, 24–26
 - 3,4-methylenedioxymethamphetamine
 - neurotoxicity and, 625–627
 - obsessive-compulsive disorders
 - abnormalities, 219–220
 - genetic studies, 222–224
- Serotonin transporter (SERT) antagonists
 - obsessive-compulsive disorders, 223
- Sertindole
 - schizophrenia therapy
 - clinical profile, 383–387
- Signal transduction mechanisms
 - psychostimulant abuse
 - chronic exposure and sensitization effects, 584–585
- Single-nucleotide polymorphisms (SNPs)
 - addictive disorders vulnerability, 457–458
 - catechol-*O*-methyl transferase
 - as schizophrenia susceptibility gene, 347–348
- schizophrenia genetics
 - DISC1 molecular interactions, 352
 - GRM3 gene, 349
 - positional candidate genes, 325
- Single-photon-emission computerized tomography (SPECT)
 - schizophrenia analysis, 257
- Site-specific microinjection studies
 - alcohol abuse
 - GABA_A benzodiazepine receptor
 - complex manipulation, 493–496
- Smoking. *See* Nicotine
- Social anxiety disorder
 - pharmacotherapy, 79–80
- Social interaction paradigm
 - 3,4-methylenedioxymethamphetamine
 - effects on, 630–631
- Social isolation
 - animal anxiety model
 - neurosteroid effects, 151–152
 - schizophrenia
 - animal models, 264
- Soluble N-ethylmaleimide-sensitive factor
 - attachment protein receptors (SNAREs)
 - dystrobrevin binding protein 1 (DTNBP1)
 - molecular interactions, 353
- "Speedball" psychostimulant combination
 - neuropeptide pharmacology and, 578–579
- Splice variants
 - corticotropin-releasing factor receptor
 - antagonists
 - receptor subunits, 182–185
- SR48692
 - psychostimulant abuse
 - neuropeptide pharmacology and, 579
- SR144528 antagonist
 - cannabinoid receptors and, 661–662
- Stimulant medications
 - psychostimulant therapy, 587–588
- Stress
 - addictive disorders and response to, 459
 - cannabinoid analgesics and, 667–668
 - neuroactive steroid interactions
 - drug abuse relapse, 159–160

- HPA axis, 153–154
 - overview, 152–153
- psychostimulant abuse
 - chronic exposure and sensitization effects, 580–585
 - neuropharmacology and, 577–578
- Striatal dopamine receptors
 - atypical antipsychotic drugs
 - clozapine D₂ receptor blockade, 414–415
 - schizophrenia dopamine hypothesis and, 287–288
- Structural studies
 - obsessive-compulsive disorders, 217–218
- Structure-activity relationships (SARs)
 - benzodiazepines, 114–116
- Substance P
 - anxiety neurobiology and, 17
- Substantia nigra
 - clozapine D₂ receptor blockade, 414–415
- Sucrose-fading technique
 - alcohol abuse studies in alcohol-preferring rats, 467–470
- Sulfate fraction
 - neuroactive steroids
 - in brain, 137
- Sulpiride
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - ventral pallidum receptor blockade, 477–480
- Sumatriptan
 - migraine management with, 764–765
 - obsessive-compulsive disorders, 234
- Susceptibility genes
 - schizophrenia
 - future research issues, 355–356
 - gene linkage studies, 324
 - molecular genetics
 - functional implications, 332–333
 - molecular interactions, 351–355
 - postmortem studies
 - overview, 343–344
 - susceptibility gene identification, 344–351
 - susceptibility gene interactions, 351–355
- Sweetened cocktail solution procedure
 - alcohol abuse studies in alcohol-preferring rats, 468–470
- Synapsin I proteins
 - dystrobrevin binding protein 1 (DTNBP1)
 - molecular interactions, 353
- Synaptosomal-associated protein 25 (SNAP25)
 - dystrobrevin binding protein 1 (DTNBP1)
 - molecular interactions, 353
- Synthesis
 - antipsychotics
 - schizophrenia pharmacotherapy, 381–382
 - endocannabinoid system, 663–664
- Systemic administration studies
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex, 492–493
- Systems biology theory
 - schizophrenia and, 255–256
- "Systems neuroscience"
 - schizophrenia dopamine hypothesis and, 288
- T*-butyl agents
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex
 - site-specific microinjection techniques, 494–496
- Δ⁹-Tetrahydrocannabinol
 - analgesic properties, 666–669
 - appetite regulation, 670–672
 - cannabinoid receptor binding, 663
 - cognition and, 669–670
 - emesis modulation, 674–675
 - endocannabinoid physiology and, 665–675
 - isolation off, 660–661
 - neurotoxicity effects, 672–673
 - prenatal development and, 664–665
 - reward, tolerance, and dependence, 675–676
- Thermoregulation
 - 3,4-methylenedioxymethamphetamine
 - effects on, 629
- Thioxanthine
 - schizophrenia therapy
 - clinical profile, 383
- THIP agonist
 - alcohol abuse studies
 - GABA_A benzodiazepine receptor complex, 488–493

- Tiagabine
 - psychostimulant abuse therapy, 588
- Tissue injury
 - transient receptor potential V1 (TRPV1)
 - receptor expression, 729–732
- Tolcapone
 - schizophrenia therapy, 389
- Tolerability
 - schizophrenia therapy
 - second-generation antipsychotics, 387–388
- Tolerance mechanisms
 - benzodiazepines, 100
 - endocannabinoids, 675–676
 - psychostimulant abuse
 - neurobiology of, 579–585
- Topiramate
 - migraine management, 766
 - psychostimulant abuse therapy, 688
- Topological analysis
 - corticotropin-releasing factor receptor
 - antagonists
 - receptor subunits, 183–185
- Tourette's disorder
 - obsessive-compulsive disorder
 - comorbidity, 216
- Tramadol
 - obsessive-compulsive disorder therapy, 234
- Transcranial magnetic stimulation (TMS)
 - obsessive-compulsive disorders, 236
- Transcriptional regulators
 - anxiety neurobiology and
 - mouse studies, 19, 22
- Transdermal nicotine patch
 - mecamylamine in conjunction with, 549
 - nicotine dependence and withdrawal
 - therapy, 546–547
 - obsessive-compulsive disorder therapy, 234
- Transient receptor potential V1 (TRPV1)
 - receptors
 - analgesic properties, 668–669
 - antagonists, 733
 - capsaicin, protons, and heat, 730–732
 - chemical activators, 732–733
 - cloning of, 728–729
 - desensitization, 735–736
 - endocannabinoid ligands, 664
 - expression, 729
 - knockout mouse models, 736
 - nociception channels, 736–737
 - sensitization, 733–735
- Transient receptor potential V2 (TRPV2)
 - nociception and, 736–737
- Transient receptor potential V3 (TRPV3)
 - nociception and, 737
- Transient receptor potential V4 (TRPV4)
 - nociception and, 737
- TRAR4 gene
 - schizophrenia molecular genetics, 328
- Treatment Episode Data Set (TEDS)
 - opiate treatment statistics, 694–696
- Tricyclic antidepressants (TCAs)
 - anxiety disorder therapy, 69–70
 - nicotine dependence and withdrawal
 - therapy, 550
 - panic disorder therapy, 78
 - psychostimulant abuse therapy, 586–587
- Trigeminal ganglion
 - migraine therapy targeting of, 760
- Trigeminovascular system
 - calcitonin gene-related peptide sites and
 - migraine therapy targeting, 761–762
- Triptans
 - migraine management with, 764–765
- Tryptophan hydroxylase
 - 3,4-methylenedioxymethamphetamine
 - effects on, 616–617
- Two-bottle water choice procedure
 - alcohol abuse studies in alcohol-preferring
 - rats, 468–470
- Tyrosine-hydroxylase
 - schizophrenia dopamine hypothesis and, 288–289
- Tyrosine kinase B (trkB)
 - anxiety neurobiology
 - receptor deficits and, 29–30
- Urocortins
 - corticotropin-releasing factor receptor
 - antagonists
 - ligand structure, 180–181
- Val66Met substitution polymorphism
 - anxiety neurobiology
 - genetic susceptibility studies, 18–19
 - schizophrenia
 - serotonin receptor 5-HT_{2A} receptor
 - and, 421–422
- Valproate
 - anxiety disorder therapy, 75
 - migraine management, 765–766

- Vanilloid receptors
 - cloning, 727–729
 - transient receptor potential V1 expression, 729
 - antagonists, 733
 - capsaicin, protons, and heat, 730–732
 - chemical activators, 732–733
 - desensitization, 735–736
 - knockout mouse models, 736
 - nociception channels, 736–737
 - sensitization, 731–735
- Varenicline
 - nicotine dependence and withdrawal therapy, 548
- Variable numbers of tandem repeats (VNTR)
 - obsessive-compulsive disorders
 - dopamine genetics, 223
- Velocardiofacial syndrome gene
 - schizophrenia
 - chromosome mapping, 346
 - COMT catabolism and, 329
- Venlafaxine
 - anxiety disorder therapy, 69
 - obsessive-compulsive disorder therapy, 234
- Ventral hippocampal lesions
 - in schizophrenia
 - animal models, 265
- Ventral pallidum
 - alcohol abuse studies, 476–480
 - dopaminergic regulation hypotheses, 478–480
 - GABA_{A1} subunit probe selectivity, 501–503
 - GABAergic modification of dopamine agonists, 497–498
- Ventral tegmental area (VTA)
 - alcohol abuse studies
 - dopaminergic receptor systems and substrates, 472–473
 - alcohol abuse studies in alcohol-preferring rats
 - control substrates in, 470
 - atypical antipsychotic drug mechanisms in
 - clozapine D₂ receptors, 414–415
 - cannabinoid receptors in, 675–676
 - integrated glutamate/dopamine
 - hypotheses of schizophrenia, 375–376
 - nicotine dependence and withdrawal
 - nicotine reinforcement substrates, 538–540
 - withdrawal substrates, 542–543
 - psychostimulant abuse
 - monoamine neuropharmacology, 571–575
 - neuropeptide pharmacology, 578–579
- Vesicular glutamate transporter
 - glutamate theory of schizophrenia
 - pathological evidence, 292
- Vesicular monoamine transporter (VMAT)
 - protein
 - dopamine hypothesis of schizophrenia, 284–285
- Visual system
 - γ -hydroxybutyric acid (GHB) effects, 634
- Vitamin D receptor (VDR)
 - anxiety neurobiology and, 32
- Vogel conflict test
 - mouse models of anxiety
 - neurosteroid effects, 145–146
- Vogel punished drinking test
 - emotionality studies in mice, 8–9
- WAY-100635 compound
 - 5-HT_{2A}-5-HT_{2C} receptor interactions, 422–424
 - nicotine dependence and withdrawal
 - withdrawal substrate specificity, 542–543
- WIN 55,212-2
 - analgesic properties, 666–668
 - convulsant effects of, 673
 - pain management and, 667–668
- Wisconsin card sort task (WCST)
 - schizophrenia dopamine hypothesis
 - genetic evidence, 286–287
- Withdrawal syndrome
 - benzodiazepines, 74
 - nicotine dependence and withdrawal
 - withdrawal neurosubstrates, 541–543
 - opiate addiction and, 699
- ZDHHC8 candidate gene
 - schizophrenia molecular genetics, 330
- Ziprasidone
 - schizophrenia therapy
 - safety and tolerability, 389
- Zolpidem
 - alcohol abuse studies
 - GABA_{A1} receptor subunit selectivity, 499

PREFACE

Neuropharmacology is the study of drugs that affect the nervous system. This includes not only the identification of neuronal drug targets but also the study of basic mechanisms of neural function that may be amenable to pharmacological manipulation. Indeed, neuropharmacological drugs are commonly used as valuable tools to discover how nerve cells function and communicate in addition to therapeutic agents for the treatment of a wide variety of neuropsychiatric disorders. In fact, drugs that are used to treat disorders of the brain and nervous system represent one of the largest groups of approved therapeutic agents. Clearly the demand for drugs to treat disorders of the nervous system will only grow in the face of an aging population. Not surprisingly, almost all major pharmaceutical corporations and many biotechnology companies have extensive drug discovery programs in neuroscience and neuropharmacology. The recent pace of research and discovery in neuropharmacology and associated therapeutics has been quite rapid, as is true for most areas of biomedical research. Given this as well as the extremely broad nature of the field, we felt that it would be timely and important to develop a comprehensive handbook of neuropharmacology that would include state-of-art reviews covering both basic principles and novel approaches for clinical therapeutics.

Our approach for the organization of this handbook was primarily translational (bench to bedside) in nature. The three book volumes consist of 10 clinical sections, each consisting of 4–7 chapters devoted to various neuropsychiatric disorders, including mood, anxiety, and stress disorders, psychosis, pain, neurodegeneration, and many others. In most cases, these sections have introductory chapters providing background information and/or basic principles prior to presenting chapters covering state-of-the-art therapeutics. Volume I also contains a large introductory section consisting of 17 chapters on basic neuropharmacological subjects and principles. These include chapters on the history of neuropharmacology as well as intercellular and intracellular signaling followed by chapters covering all of the major neurotransmitter systems and other important signaling molecules, such as ion channels and transporters. Our objective for this project was to create a high-level reference work that will be useful to all practitioners of neuropharmacology ranging from graduate students, academicians, and clinicians to industrial scientists working in drug discovery. These volumes will be part of the John Wiley & Sons major reference work program and will be published online as well as in print. The online version of this handbook is expected to undergo frequent updates and additions in order to maintain its cutting-edge status.

The editors would like to thank all of the chapter contributors for their hard work and commitment to this project. We would also like to thank our managing editor, Jonathan Rose, at John Wiley & Sons for all of the valuable assistance that he has provided.

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Contents

Preface	xi
Contributors	xiii

VOLUME 1

PART I BASIC NEUROPHARMACOLOGY	1
Chapter 1 Soup or Sparks: The History of Drugs and Synapses	3
<i>William Van der Kloot</i>	
Chapter 2 Synaptic Transmission: Intercellular Signaling	39
<i>J. David Jentsch and Robert H. Roth</i>	
Chapter 3 Synaptic Transmission: Intracellular Signaling	59
<i>R. Benjamin Free, Lisa A. Hazelwood, Yoon Namkung Michele L. Rankin, Elizabeth B. Rex, and David R. Sibley</i>	
Chapter 4 Neuronal Nicotinic Receptors: One Hundred Years of Progress	107
<i>Kenneth J. Kellar and Yingxian Xiao</i>	
Chapter 5 Muscarinic Acetylcholine Receptors	147
<i>Jürgen Wess</i>	
Chapter 6 Norepinephrine/Epinephrine	193
<i>Megan E. Kozisek and David B. Bylund</i>	
Chapter 7 Dopaminergic Neurotransmission	221
<i>John A. Schetz and David R. Sibley</i>	
Chapter 8 Serotonin Systems	257
<i>John A. Gray and Bryan L. Roth</i>	
Chapter 9 Neuropharmacology of Histamine in Brain	299
<i>Raphaël Faucard and Jean-Charles Schwartz</i>	

Chapter 10	Ionotropic Glutamate Receptors	365
	<i>David Bleakman, Andrew Alt, David Lodge, Daniel T. Monaghan, David E. Jane, and Eric S. Nisenbaum</i>	
Chapter 11	Metabotropic Glutamate Receptors	421
	<i>James A. Monn, Michael P. Johnson, and Darryle D. Schoepp</i>	
Chapter 12	Pharmacology of the GABA_A Receptor	465
	<i>Dmytro Berezhnoy, Maria C. Gravielle, and David H. Farb</i>	
Chapter 13	Metabotropic GABA Receptors	569
	<i>Martin Gassmann and Bernhard Bettler</i>	
Chapter 14	Voltage-Gated Ion Channels	617
	<i>Alex Fay, Patrick C. G. Haddick, and Lily Yeh Jan</i>	
Chapter 15	Neuropeptides	669
	<i>Fleur L. Strand</i>	
Chapter 16	Neurotransmitter Transporters	705
	<i>Jia Hu, Katherine Leitzell, Dan Wang, and Michael W. Quick</i>	
Chapter 17	Gaseous Signaling: Nitric Oxide and Carbon Monoxide as Messenger Molecules	743
	<i>Kenny K. K. Chung, Valina L. Dawson, and Ted M. Dawson</i>	
PART II	MOOD DISORDERS	763
Chapter 18	Neurobiology and Treatment of Depression	765
	<i>Alexander Neumeister, Dennis S. Charney, Gerard Sanacora, and John H. Krystal</i>	
Chapter 19	Neurotrophic Factors in Etiology and Treatment of Mood Disorders	789
	<i>Ronald S. Duman</i>	
Chapter 20	Antidepressant Treatment and Hippocampal Neurogenesis: Monoamine and Stress Hypotheses of Depression Converge	821
	<i>Alex Dranovsky and René Hen</i>	
Chapter 21	Neuroendocrine Abnormalities in Women with Depression Linked to the Reproductive Cycle	843
	<i>Barbara L. Parry, Charles J. Meliska, L. Fernando Martinez, Eva L. Maurer, Ana M. Lopez, and Diane L. Sorenson</i>	
Chapter 22	Neurobiology and Pharmacotherapy of Bipolar Disorder	859
	<i>R. H. Belmaker, G. Agam, and R. H. Lenox</i>	
Index		877
Cumulative Index		915

VOLUME 2

PART I	ANXIETY AND STRESS DISORDERS	1
Chapter 1	Neurobiology of Anxiety	3
	<i>Miklos Toth and Bojana Zupan</i>	
Chapter 2	Pharmacotherapy of Anxiety	59
	<i>Jon R. Nash and David J. Nutt</i>	
Chapter 3	Benzodiazepines	93
	<i>Hartmut Lüddens and Esa R. Korpi</i>	
Chapter 4	Neuroactive Steroids in Anxiety and Stress	133
	<i>Deborah A. Finn and Robert H. Purdy</i>	
Chapter 5	Emerging Anxiolytics: Corticotropin-Releasing Factor Receptor Antagonists	177
	<i>Dimitri E. Grigoriadis and Samuel R. J. Hoare</i>	
Chapter 6	Neurobiology and Pharmacotherapy of Obsessive-Compulsive Disorder	215
	<i>Judith L. Rapoport and Gale Inoff-Germain</i>	
PART II	SCHIZOPHRENIA AND PSYCHOSIS	249
Chapter 7	Phenomenology and Clinical Science of Schizophrenia	251
	<i>Subroto Ghose and Carol Tamminga</i>	
Chapter 8	Dopamine and Glutamate Hypotheses of Schizophrenia	283
	<i>Bitá Moghaddam and Houman Homayoun</i>	
Chapter 9	Molecular Genetics of Schizophrenia	321
	<i>Liam Carroll, Michael C. O'Donovan, and Michael J. Owen</i>	
Chapter 10	Postmortem Brain Studies: Focus on Susceptibility Genes in Schizophrenia	343
	<i>Shiny V. Mathew, Shruti N. Mitkus, Barbara K. Lipska, Thomas M. Hyde, and Joel E. Kleinman</i>	
Chapter 11	Pharmacotherapy of Schizophrenia	369
	<i>Zafar Sharif, Seiya Miyamoto, and Jeffrey A. Lieberman</i>	
Chapter 12	Atypical Antipsychotic Drugs: Mechanism of Action	411
	<i>Herbert Y. Meltzer</i>	

PART III	SUBSTANCE ABUSE AND ADDICTIVE DISORDERS	449
Chapter 13	Introduction to Addictive Disorders: Implications for Pharmacotherapies	451
	<i>Mary Jeanne Kreek</i>	
Chapter 14	Dopaminergic and GABAergic Regulation of Alcohol-Motivated Behaviors: Novel Neuroanatomical Substrates	465
	<i>Harry L. June and William J. A. Eiler II</i>	
Chapter 15	Nicotine	535
	<i>August R. Buchhalter, Reginald V. Fant, and Jack E. Henningfield</i>	
Chapter 16	Psychostimulants	567
	<i>Leonard L. Howell and Heather L. Kimmel</i>	
Chapter 17	MDMA and Other “Club Drugs”	613
	<i>M. Isabel Colado, Esther O’Shea, and A. Richard Green</i>	
Chapter 18	Marijuana: Pharmacology and Interaction with the Endocannabinoid System	659
	<i>Jenny L. Wiley and Billy R. Martin</i>	
Chapter 19	Opiates and Addiction	691
	<i>Frank J. Vocci</i>	
PART IV	PAIN	707
Chapter 20	Neuronal Pathways for Pain Processing	709
	<i>Gavril W. Pasternak and Yahong Zhang</i>	
Chapter 21	Vanilloid Receptor Pathways	727
	<i>Makoto Tominaga</i>	
Chapter 22	Opioid Receptors	745
	<i>Gavril W. Pasternak</i>	
Chapter 23	Advent of A New Generation of Antimigraine Medications	757
	<i>Ana Recober and Andrew F. Russo</i>	
	Index	779
	Cumulative Index	817
VOLUME 3		
PART I	SLEEP AND AROUSAL	1
Chapter 1	Function and Pharmacology of Circadian Clocks	3
	<i>Gabriella B. Lundkvist and Gene D. Block</i>	

Chapter 2	Melatonin Receptors in Central Nervous System	37
	<i>Margarita L. Dubocovich</i>	
Chapter 3	Narcolepsy: Neuropharmacological Aspects	79
	<i>Seiji Nishino</i>	
Chapter 4	Hypocretin/Orexin System	125
	<i>J. Gregor Sutcliffe and Luis de Lecea</i>	
Chapter 5	Prokineticins: New Pair of Regulatory Peptides	163
	<i>Michelle Y. Cheng and Qun-Yong Zhou</i>	
Chapter 6	Sedatives and Hypnotics	177
	<i>Keith A. Wafford and Paul J. Whiting</i>	
PART II	DEVELOPMENT AND DEVELOPMENTAL DISORDERS	201
Chapter 7	Regulation of Adult Neurogenesis	203
	<i>Heather A. Cameron</i>	
Chapter 8	Neurotrophic Factors	221
	<i>Franz F. Hefti and Patricia A. Walicke</i>	
Chapter 9	Neurotrophins and Their Receptors	237
	<i>Mark Bothwell</i>	
Chapter 10	Tourette's Syndrome and Pharmacotherapy	263
	<i>Pieter Joost van Watum and James F. Leckman</i>	
Chapter 11	Neuropharmacology of Attention-Deficit/Hyperactivity Disorder	291
	<i>Paul E. A. Glaser, F. Xavier Castellanos, and Daniel S. Margulies</i>	
Chapter 12	Psychopharmacology of Autism Spectrum Disorders	319
	<i>Adriana Di Martino, Steven G. Dickstein, Alessandro Zuddas, and F. Xavier Castellanos</i>	
PART III	NEURODEGENERATIVE AND SEIZURE DISORDERS	345
Chapter 13	Stroke: Mechanisms of Excitotoxicity and Approaches for Therapy	347
	<i>Michael J. O'Neill, David Lodge, and James McCulloch</i>	
Chapter 14	Epilepsy: Mechanisms of Drug Action and Clinical Treatment	403
	<i>William H. Theodore and Michael A. Rogawski</i>	
Chapter 15	Pharmacotherapy for Traumatic Brain Injury	443
	<i>Donald G. Stein and Stuart W. Hoffman</i>	
Chapter 16	Dementia and Pharmacotherapy: Memory Drugs	461
	<i>Jerry J. Buccafusco</i>	

Chapter 17	Pharmacotherapy and Treatment of Parkinson's Disease	479
	<i>Wing Lok Au and Donald B. Calne</i>	
Chapter 18	Parkinson's Disease: Genetics and Pathogenesis	523
	<i>Claudia M. Testa</i>	
Chapter 19	Invertebrates as Powerful Genetic Models for Human Neurodegenerative Diseases	567
	<i>Richard Nass and Charles D. Nichols</i>	
PART IV	NEUROIMMUNOLOGY	589
Chapter 20	Myelin Lipids and Proteins: Structure, Function, and Roles in Neurological Disorders	591
	<i>Richard H. Quarles</i>	
Chapter 21	Pharmacology of Inflammation	621
	<i>Carmen Espejo and Roland Martin</i>	
Chapter 22	Pharmacological Treatment of Multiple Sclerosis	671
	<i>B. Mark Keegan</i>	
Chapter 23	Novel Therapies for Multiple Sclerosis	683
	<i>Martin S. Weber and Scott S. Zamvil</i>	
Chapter 24	Neuropharmacology of HIV/AIDS	693
	<i>Sidney A. Houff and Eugene O. Major</i>	
PART V	EATING AND METABOLIC DISORDERS	731
Chapter 25	Leptin: A Metabolic Perspective	733
	<i>Dawn M. Penn, Cherie R. Rooks, and Ruth B. S. Harris</i>	
Chapter 26	Ghrelin: Structural and Functional Properties	765
	<i>Birgitte Holst, Kristoffer Egerod, and Thue W. Schwartz</i>	
Chapter 27	Mechanisms Controlling Adipose Tissue Metabolism by the Sympathetic Nervous System: Anatomical and Molecular Aspects	785
	<i>Sheila Collins, Renato H. Migliorini, and Timothy J. Bartness</i>	
Chapter 28	Antiobesity Pharmacotherapy: Current Treatment Options and Future Perspectives	815
	<i>Yuguang Shi</i>	
Index		845
Cumulative Index		881

1

FUNCTION AND PHARMACOLOGY OF CIRCADIAN CLOCKS

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1.1	Functional Importance of Circadian Rhythms	4
1.2	Properties of Circadian Clocks	4
1.2.1	Circadian Rhythms	4
1.2.2	Temperature Sensitivity	5
1.2.3	“Entrainment” Properties	5
1.2.4	Aschoff’s Rules	5
1.2.5	Aftereffects	6
1.3	Structure of Circadian Timing System in Nonmammalian Models	6
1.3.1	Molecular Basis of Rhythmicity: <i>Drosophila</i>	7
1.3.2	Drug Effects in <i>Drosophila</i>	7
1.3.3	Cellular Basis of Rhythmicity: Molluscan Retina	8
1.3.4	Circadian Rhythmicity: A Cell Autonomous Property	8
1.3.5	Synchronization and Modulation of Clock Neurons	8
1.3.6	Ionic Currents Involved in Circadian Rhythmicity	9
1.3.7	System-Level Properties: Cockroach	10
1.4	Structure of Circadian Timing System in Mammals	10
1.4.1	Suprachiasmatic Nucleus: A Central Circadian Timer	10
1.4.2	Circadian Rhythms: Generated by Molecular Feedback Loops	11
1.4.3	Role of Membrane Activity in Circadian Rhythm Generation	12
1.4.4	Output Pathways of SCN	12
1.5	Agents that Affect Period and Phase of SCN	13
1.6	Chronopharmacology	15
1.6.1	Affective Disorders and Lithium	15
1.6.2	Antidepressants and Effects on Sleep	17
1.6.3	Antidepressants and Circadian Rhythms	18
1.6.4	Benzodiazepines	20
1.6.5	Inflammatory Molecules	21
1.6.6	Cytokines and Disturbances in Circadian Sleep–Wake Rhythms	21
1.7	Concluding Remarks	23
	References	24

1.1 FUNCTIONAL IMPORTANCE OF CIRCADIAN RHYTHMS

Biological molecules interact in space and in time. Understanding spatial relationships in biology has been fundamental to our comprehension of how function follows form. The study of time in biology (“temporal biology”) is a more recent area of inquiry; however, it has become increasingly clear that timing is a fundamental attribute of living systems. Organisms have the capacity to generate oscillations in physiology and behavior over a wide time domain. Subsets of biological oscillations have period lengths that match major environmental cycles and consequently can be used as internal chronometers matching the external periodicity. Most common among the biological rhythms are circadian rhythms, periodicities of approximately 24 h.

Biological clocks, expressing periodicities within the circadian domain, exert a profound effect on behavior and physiology, creating a “day within” [1] that allows organisms to prepare in advance of environmental changes. In higher animals, including mammals, biological timers orchestrate cycles of sleep and wakefulness, rhythmicity in major hormonal systems, as well as periodic fluctuations in cognitive performance, blood pressure, and response to drug administration [2]. Circadian clocks also play a less obvious but sophisticated role in the solar navigation behavior displayed by species of bees, birds, reptiles, and amphibians. Honeybees, for example, rely on the biological clock to use the sun as a compass for navigation [3]. Similar to bees, several species of birds use the position of the sun as a compass in navigating terrain. A properly set biological clock is critical for interpreting the sun’s azimuth and a bird synchronized to an altered light schedule will make errors in flight orientation proportional to the number of hours the biological clock is set away from local time [4].

Circadian clocks also play an unexpected but critical role in the seasonal timing of flowering in many plants and reproductive cycles in animals. Although some animals possess approximately 12-month timers (circannual rhythms), other animals rely on a circadian clock to measure day length or night length. Some mammals, including several rodent species, exhibit seasonal reproductive rhythms that use a circadian clock to generate appropriate seasonal responses. Such animals have an underlying circadian “sensitivity rhythm” to light. Although the exact mechanism is uncertain, the circadian sensitivity rhythm allows the system to determine whether the seasonal light cycle consists of short or long days [5].

1.2 PROPERTIES OF CIRCADIAN CLOCKS

1.2.1 Circadian Rhythms

When measured under constant environmental conditions, most higher organisms exhibit activity–rest cycles as well as a multitude of rhythmic physiological parameters. In humans there is a pronounced sleep–wakefulness rhythm, a rhythm in body temperature, and rhythms in cognitive performance [6]. Little escapes the influence of the circadian timer. Much of the research on circadian clocks in mammals has been performed in rodent models. Many rodents exhibit precise rhythms in wheel-running activity, and this has frequently been used as a convenient rhythm for measuring the properties of the circadian timing system [7]. If the running

wheel is housed in constant darkness, most rodent species will exhibit a periodicity slightly less than 24 h. Such rhythms are referred to as “free-running” and are the expression of the circadian timing system without being influenced by environmental synchronizing signals.

1.2.2 Temperature Sensitivity

Not surprisingly, in order to be a faithful chronometer, the biological clock must not change frequency at different environmental temperatures. In mammals there is obvious thermal buffering due to active homeostatic control of body temperature. Nonetheless, both in homoeothermic and poikilothermic organisms, the circadian clock is relatively insensitive to ambient temperatures within the normal environmental range [8]. The Q_{10} measurements (response of the clock period to 10-degree temperature changes) are ‘close to 1, indicating near constancy’ of period over a 10-degree change. In contrast, many biochemical reactions have Q_{10} ’s of 2 or greater (doubling or greater of reaction rate). It is not true, however, that circadian clocks are temperature insensitive. Many organisms respond to a temperature step with a shift in the phase of the rhythm and many clocks can be synchronized by periodic temperature cycles [9]. It is evident that the relative temperature independence that is observed at any constant temperature does not reflect insensitivity to temperature but rather some form of temperature compensatory mechanism.

1.2.3 “Entrainment” Properties

If rodents are maintained on 24-hr light–dark (LD) cycles, the circadian rhythm in locomotor activity adopts the period of the 24-h light schedule. This process synchronizes (i.e., “entrains”) the circadian clock to the 24-h solar cycle and, importantly, sets the phase of the clock to “local time” (activity occurs in relation to the local day–night cycle). Except for measuring the lapse of time (e.g., a “stopwatch”), clocks must be set to local time to be truly useful. There are several phenomenological models that have helped to explain the synchronization, or entrainment, process. Entrainment appears to occur primarily through rapid changes in the phase of the clock brought about by light–dark transitions in the light cycle [10, 11]. The so-called nonparametric model for entrainment postulates that the transition at dawn leads to a rapid phase advance of the clock and the transition to dusk leads to a phase delay [10, 12]. The two phase shifts are typically not equal in magnitude and the net phase shift equals the difference between the 24-h light cycle and the period of the biological clock. Elegant in its simplicity, the model has been useful in focusing on the importance of rapid phase adjustments on the entrainment process. These authors recognized that entrainment was more complex than simply rapid phase adjustments at dawn and dusk, and more recent work has helped inform the model by taking into account the more subtle effect of light on the clock’s period [13].

1.2.4 Aschoff’s Rules

Although the free-running period is under genetic control and varies among species, the precise period may vary depending upon constant conditions and the prior light history. Jürgen Aschoff explored the effects of constant light on the free-running

period [14]. Numerous studies in a wide range of species led to a set of empirical generalities about the effects of different-intensity light on the free-running rhythm. Known as *Aschoff's rules*, these observations noted that diurnal and nocturnal animals respond differently to increasing the intensity of constant light [2]. Nocturnal animals tend to exhibit longer free-running periods as the intensity of light increases whereas the rhythms of diurnal animals tended to shorten in brighter constant light. Also the ratio of activity time compared to rest time increased in diurnal animals as light intensity increased and decreased in nocturnal animals. Finally, in general but with exceptions, diurnal animals tend to have free-running periods longer than 24 h in constant darkness and nocturnal animals tend to have periods of less than 24 h. These observations made it clear that the free-running period of locomotor activity in a rodent was a function of the constant photic/nonphotic environment to which it was exposed. It has been proposed that the change in free-running period in response to long light pulses is probably part of the mechanism by which light cycles act to adjust the free-running period to match more closely the period of the light cycle [13, 15].

1.2.5 Aftereffects

In addition to changes in free-running period associated with the current photic environment, the biological clock may be influenced by the prior history [16]. Several types of history-dependent effects are observed, depending upon the species. Mice or hamsters exposed to short or long photoperiods (representing long spring or short winter days) exhibit different durations of locomotor activity and different free-running periods when subsequently placed into constant darkness [12, 16]. In addition, animals exposed to light cycle periods either shorter or longer than 24 h subsequently exhibit free-running periods in constant darkness positively correlated with the prior light cycle period. Thus, both the free-running period and the waveform of the circadian rhythm are influenced by prior photic history. Although the function of such “aftereffects” is not fully understood, it has been proposed that this mechanism may be centrally involved in period adjustments that contribute to stable entrainment [10].

1.3 STRUCTURE OF CIRCADIAN TIMING SYSTEM IN NONMAMMALIAN MODELS

Much of what we know about the mechanisms underlying circadian timing comes from studies on nonmammalian models. While organisms in nearly all phyla have been investigated, a few models have emerged as preeminent for study at different levels of circadian organization. At the molecular level, *Drosophila* (fruitfly) has provided the most detailed and satisfying model of the molecular components that interact to generate a 24-h periodicity. Marine mollusks, notably *Aplysia* (sea hare) and *Bulla* (cloudy bubble snail), have provided cellular/biophysical mechanisms involved in the synchronization and expression of circadian rhythms. Additional models, such as the cockroach, have yielded important insights into the system-level organization underlying circadian rhythms. Much of what we have learned from these model systems has informed similar discoveries in mammals.

1.3.1 Molecular Basis of Rhythmicity: *Drosophila*

Although many aspects of circadian timing have been studied in insects, perhaps the greatest contribution of insects to understanding circadian rhythmicity comes from molecular/genetic studies in *Drosophila*. Early phenomenological analysis in *Drosophila* provided the foundations for understanding the synchronization process by light cycles [12]. However, the focus of research and pace of discovery was fundamentally changed by the isolation of long-period, short-period, and arrhythmic mutants, all of which turned out to be alleles of the same gene, referred to as *period* [17]. Subsequent mutagenesis, isolation and cloning yielded several additional “clock genes” including *timeless*, *doubletime*, *clock*, *cycle*, and *cryptochrome* [18]. Although the *Drosophila* clock model continues to develop, a working hypothesis has been elaborated in which clock genes and their products form inhibitory and excitatory feedback loops which together generate a stable circadian period [18, 19].

1.3.2 Drug Effects in *Drosophila*

The fruit fly, *Drosophila melanogaster*, represents a “simple” genetic model in which to study pathways modulating responses to drugs of abuse. Flies respond to a number of drugs of abuse with motor responses that strikingly resemble those seen in vertebrate models: Exposure to ethanol leads to transient motor activation followed by sedation [20–23]. Exposure to the psychomotor stimulant cocaine leads to extensive stereotypic motor patterns, including reflexive grooming, locomotion, and at high doses hyperactive behaviors, including wing buzzing, that are termed “seizures” in the fly literature [21, 24]. Additionally, flies can show enhanced responses to repeated doses of cocaine, a process called sensitization [24], and tolerance to repeated doses of ethanol [25, 26]. Tolerance and sensitization are responses seen in animal models and humans that are thought to reflect the altered homeostatic responses that develop with repeated drug exposures. Sensitization is thought to contribute to the addictive process [27, 28] and is rather commonly used as an indirect correlate of craving.

One obvious question when using the fly as a model for these complex animal behaviors regards the relevance of the model. It is now clear that many similar pathways are involved relative to vertebrates, including dopamine and/or serotonin, which modulate responses to cocaine, ethanol, and nicotine [21, 29], and G-protein and protein kinase A signaling, which modulate responses to cocaine [29, 30]. This, along with work showing cyclic adenosine monophosphate (cAMP) pathway involvement in ethanol responses [20, 30, 31], further increases the parallels between the signaling pathways underlying responses to drugs of abuse between flies and higher vertebrates [32].

The real interest in the fly as a model is to define novel pathways that would not have necessarily been uncovered in vertebrates. One pathway pioneered in the fly is the involvement of the circadian gene pathway in modulating responses to cocaine, particularly the sensitization to repeated cocaine exposures, which requires most but not all of the circadian genes in the fly [33–35]. This observation now has validation in mouse knockouts [36]. Mice have three *period* genes, of which *mper2* is the major circadian *per* gene. The *per2* knockouts show somewhat enhanced conditioned place preference (CPP) and locomotor sensitization, but *mper1* knockouts show loss of

both CPP and sensitization. Given that *mper2* knockouts show a more severe circadian phenotype than *mper1* [37–39], this implies a separation of circadian and drug reward roles for these genes. In addition, *clock* mutant mice demonstrate enhanced CPP to cocaine, and enhanced excitability of ventral tegmental area (VTA) dopamine neurons [40]. Given that the VTA–nucleus accumbens dopamine tract is not central to circadian rhythmicity in the mouse, this again implies noncircadian functions of these circadian genes in function of the reward pathways.

1.3.3 Cellular Basis of Rhythmicity: Molluscan Retina

The eye of the opisthobranch mollusks, *Aplysia* and *Bulla*, contain circadian pacemakers [41, 42]. The molluscan eye model has provided many firsts in chronobiological research. As an example, mollusks led the way in confirming the cell autonomous nature of circadian pacemakers within the nervous system [46]. The presence of “chronomodulators,” such as serotonin, (5-HT) in the regulation of the pacemaker phase was first revealed in *Aplysia*. The *Aplysia* and *Bulla* mollusks provided the first reliable in vitro model for evaluating the effects of pharmacological agents on the circadian clock [43]. Intracellular recordings in molluscan clock neurons provided important information about the biophysical and intracellular processes mediating pacemaker synchronization and expression. Mollusks also provided the first in vitro models for studying circadian multioscillator coupling. Impressively, much of what has been learned from mollusks has proven directly relevant to the mammalian timing system.

1.3.4 Circadian Rhythmicity: A Cell Autonomous Property

The first demonstration that individual neurons could act as circadian pacemakers was accomplished in the molluscan retina. Experiments in which the *Bulla* retina was surgically reduced localized the circadian clock to a group of approximately 100 pacemaker neurons located at the base of the retina [44]. The demonstration of the cell autonomous nature of neuronal circadian pacemakers was accomplished by taking advantage of the fact that the pacemaker neurons of the *Bulla* retina, referred to as BRNs (basal retinal neurons), exhibit a pronounced (up to 50%) rhythm in membrane conductance with membrane conductance low during the subjective day when compared with the subjective night [45]. BRNs, either in dispersed cell culture or when completely isolated in microwell dishes, exhibited a similar circadian rhythm in conductance [46]. The persistence of daily membrane conductance changes in isolated neurons confirmed that circadian rhythm generation is a property of individual neurons. This observation was later confirmed in dispersed *Aplysia* neurons, although the cells were never fully isolated [47].

1.3.5 Synchronization and Modulation of Clock Neurons

The identification of “clock neurons” in *Bulla* and *Aplysia* opened up important opportunities for the study of the mechanisms underlying pacemaker synchronization and expression. A large number of studies by several laboratories led to a simple hypothesis that phase control of the retinal clock is effected by changes in membrane

potential and modulation of a transmembrane calcium (Ca^{2+}) flux [48]. In this model, light cycles lead to membrane depolarization and phase adjustments during the early and late night. Another class of entrainment stimuli, Phe-Met-Arg-Phe-amine (FRMF)-amide (Phe-Met-Arg-Phe- NH_2) in *Bulla* [49] and 5-HT and cAMP in *Aplysia*, [50], generate phase shifts that are displaced by 180° on the time axis compared with light-induced shifts; that is, phase delays occur in the early subjective day and phase advances occur in the late subjective day. These treatments lead to membrane hyperpolarization and a reduction of Ca^{2+} influx presumably due to the reduction of impulse production during the subjective day. The model is attractive in that it proposes that all synchronization signals to the clock converge on a single process, an increase or decrease in a transmembrane Ca^{2+} flux. In addition to acting as phase-shifting agents, both 5-HT and FMRF-amide can modulate light-induced phase shifts [49, 51]. Serotonin's action can be complex, both attenuating and amplifying the effects of light, depending upon the phase at which it is present [52].

1.3.6 Ionic Currents Involved in Circadian Rhythmicity

Pacemaker expression involves rhythmic modulation of potassium (K^+) conductance(s). The ability to make intracellular recordings from *Bulla* pacemaker neurons provided an opportunity to study circadian modulation in membrane phenomena. *Bulla* pacemaker neurons exhibit a circadian rhythm in the membrane potential [53]. Cells are relatively hyperpolarized during the subjective night and depolarized at subjective dawn. The membrane potential rhythm is associated with a rhythm in membrane conductance. Membrane conductance is higher during the subjective night when the BRNs are hyperpolarized [45, 48]. Application of tetraethylammonium (TEA) returns the subjective night conductance levels to subjective day time levels suggesting that K^+ channels play a critical role in the circadian rhythm in membrane conductance [46].

More recently, attempts have been made to identify specific potassium channels that are being modulated by the circadian pacemaker [54]. Three components of outward K^+ current have been identified: a transient outward current (I_A), a Ca^{2+} -dependent K^+ current ($I_{\text{K(Ca)}}$), and a sustained Ca^{2+} -independent current including the delayed rectifier, I_K . Whole-cell currents recorded before and after the projected dawn and normalized to the cell capacitance reveal a circadian modulation in the delayed rectifier current (I_K). However, the I_A and the $I_{\text{K(Ca)}}$ currents are not affected by the circadian pacemaker. The kinetics of the delayed rectifier suggest that it is not responsible for the rise in membrane potential near subjective dawn. Thus, other as-yet-unidentified K^+ conductances probably play this important role.

Similar results have been obtained with pacemaker cells in the retina of *Aplysia californica* [55]. *Aplysia* retinal neurons exhibit a decrease in the amplitude of a delayed rectifier current, measured in dispersed retinal cells, recorded at subjective dawn. As with the study in *Bulla*, experiments in *Aplysia* find no evidence for a circadian rhythm in I_A .

Perhaps the most satisfying and surprising outcome of the work on the molluscan eye is the ongoing discovery of how well conserved the cellular/biophysical circadian mechanisms are between the mollusks and mammals. With both the synchronization and expression pathways, similar ionic fluxes, conductances, and neuromodulatory pathways appear to be involved in regulation of the circadian system (see below).

1.3.7 System-Level Properties: Cockroach

Circadian systems are multioscillator in composition. At the level of pacemaker structures, such as the retina of mollusks or the suprachiasmatic nucleus (SCN) of the mammal, there are many neurons that are independently capable of generating a circadian periodicity. There is also the issue of paired pacemaker structures, by virtue of the fact that clocks can be located in eyes or in brain structures that are bilaterally symmetric. Beyond symmetric clock loci, there is evidence that many tissues can exhibit some level of rhythmicity, even when there are dominant tissues that serve as pacemakers. This complexity leads us to hypothesize the presence of both bilateral and hierarchical oscillator coupling relationships. Again, nonmammalian models, especially the cockroach, have given us significant insight into the organizational properties of circadian systems.

In the cockroach, the primary clock controlling activity–rest cycles is located within the optic lobe [56]. The clocks in the two optic lobes are coupled to one another. First, the activity–rest cycle is lengthened slightly when either lobe is removed, suggesting that the two clocks mutually accelerate one another when both are present [57]. More directly, if the clock of one lobe is delayed by several hours by cooling the lobe with a localized cooling probe, the other lobe will cause the cooled lobe to advance, while it is itself delayed [58]. The data suggest that the two lobes are mutually coupled and lead to a free-running period that is slightly shorter than either individual lobe. Beyond mutual entrainment of the bilaterally symmetric optic lobes, there appears to be a damped circadian oscillator that persists in the absence of the central optic lobe clocks. Lobeless animals, although arrhythmic on light cycles, can be driven to rhythmicity by temperature cycles [59]. The fact that the phase relationship between the locomotor rhythm and the temperature cycle changes as a function of the period of the thermocycle indicates the presence of a highly damped circadian oscillator. A model has emerged of central timers in the optic lobes that drive damped circadian oscillators in thoracic ganglia. The damped oscillators are more directly responsible for controlling the activity–rest cycle. The basic model of bilaterally symmetric central clocks controlling behavior and physiology through peripheral, perhaps highly damped clocks is an organizational principle that appears to be quite general, including mammals.

1.4 STRUCTURE OF CIRCADIAN TIMING SYSTEM IN MAMMALS

1.4.1 Suprachiasmatic Nucleus: A Central Circadian Timer

There are few examples in neuroscience research where it has been possible to unambiguously identify a restricted portion of the mammalian central nervous system as the locus of the generation and control of a complex behavior. The SCN is such a site. The SCN is a bilaterally symmetric structure lying just above the optic chiasm (Fig. 1.1). Each nuclei consists of approximately 10,000 neurons packed into a volume of about 0.5 mm^3 [60, 61]. The discovery of the SCN occurred with the efforts of two laboratories focused on identifying the pathways involved in synchronization of the circadian clock by light cycles, one laboratory looking for pathways controlling the circadian rhythm in drinking behavior [62], the other looking at pathways involved in adrenal corticosterone rhythmicity [63]. Both discovered that lesions placed above the optic chiasm led to disruption of the respective circadian

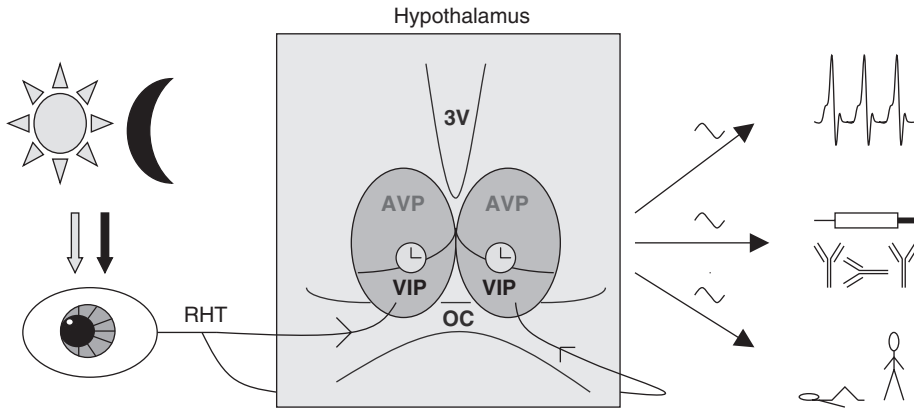


Figure 1.1 Mammalian circadian system. The internal master clock, located in the SCN in the hypothalamus in close vicinity to the third ventricle (3V), is primarily synchronized by the environmental light–dark cycle via a monosynaptic pathway in the retinohypothalamic tract (RHT). Retinal afferents reach the optic chiasm (OC) and the ventral “core” region of the SCN that contains vasoactive intestinal polypeptide (VIP). The dorsal “shell” region contains vasopressin (AVP) where most efferents to other brain regions are located. Activities of individual single-oscillator neurons are synchronized in the SCN synaptic network, giving a circadian output that drives physiological rhythms such as spontaneous neuronal activity, drug susceptibility, immune functions, and the rest–activity cycle. (See color insert.)

rhythms. The central importance of the region was firmly established by a number of experiments in which circadian rhythms in electrical activity were recorded from this region when isolated either with knife cuts from the rest of the brain [64] or by removing the SCN and recording from an SCN brain slice in vitro [65]. These experiments demonstrated that the SCN was intrinsically rhythmic and was presumably responsible for driving rhythmicity in other regions of the brain. An unambiguous demonstration of the importance of the SCN in controlling behavior occurred through transplantation experiments in which the SCN of fetal animals was transplanted into the third ventricle of the brains of adult animals with prior lesions to their SCN [66]. Rhythmicity was restored, and critically the host took on the period of the donor’s SCN. This was demonstrated through the use of a mutant hamster (*Tau* mutant) with an unusually short periodicity. Wild-type hamsters receiving the *Tau* mutant SCN exhibited short free-running periods whereas *Tau* mutant hamsters receiving the wild-type SCN exhibited longer periods. This provided an unambiguous demonstration that the SCN is responsible for generating and, to a large extent, setting the period of the animal’s activity rhythm.

1.4.2 Circadian Rhythms: Generated by Molecular Feedback Loops

Circadian rhythm generation is a property of individual neurons [46, 67]. We now know that several genes and their protein products are central to the generation of circadian rhythms. Much of the work in mammals has been informed by studies in *Drosophila* where there is similarity in underlying molecular mechanisms.

In mammals, the timing cycle is complex, with several feedback loops both positive and negative [68]. A core element in generating oscillators is the negative-feedback

loop involving a small group of genes and their protein products. This timing cycle is driven and the period determined primarily by a negative feedback loop with delay. A completed cycle involves the following steps [69]. The proteins CLOCK and BMAL1 form protein heterodimers. The CLOCK/BMAL1 complex enters the nucleus. The CLOCK/BMAL1 binds to E-box enhancer elements of *pers*, *crys*, and *Rev-Erb- α* . The binding leads to the expression of these clock genes and synthesis of the proteins PER, CRY, and REV-ERB- α . PER and CRY form homodimers and heterodimers in the cytoplasm and, when dimerized, enter the nucleus. At the same time, PER is continually phosphorylated by CKI-EPSILON, which leads to degradation, thus slowing the entry of PER into the nucleus. PER:CRY and CRY:CRY represses CLOCK:BMAL1-driven transcription of *pers* and *crys*. Thus, the negative-feedback loop is completed, as the protein products inhibit their own transcription. The long time constants for the transcriptional/translational rhythm are probably generated from several sources, including the phosphorylation of PER leading to its degradation and slowing of nuclear entry, PER:TIM's action on repressing *Per* transcription, and CRY protein inhibition of H3 histone acetylation by P300.

1.4.3 Role of Membrane Activity in Circadian Rhythm Generation

Membrane phenomena, including membrane potential, electrical impulses, and voltage-controlled ionic fluxes, have generally been considered as components of the “input pathway” and “output pathway” by which a transcription/translation-based clock receives temporal information from the environment and transmits rhythmic signals to other oscillators within the tissue and beyond. This long-held view has been challenged from time to time [70]. Perhaps the most direct challenge comes from studies in *Drosophila* [71–73] which revealed that when lateral neurons, circadian pacemaker neurons, are electrically silenced by expression of additional potassium channels, the molecular clockwork and the behavior become arrhythmic. The findings in *Drosophila* have brought new attention and focus to a possible role for membrane potential and associated transmembrane ionic fluxes in rhythm generation. Recent experiments conducted in our laboratory [74] reveal a critical role for a daily transmembrane Ca^{2+} flux in sustaining *mpc1* rhythmicity in the SCN and peripheral tissues. The precise relationship between membrane currents, Ca^{2+} oscillations, and molecular feedback loops is currently unclear.

1.4.4 Output Pathways of SCN

In order for the SCN to perform work for the organism as a central timer, it must influence other brain regions and peripheral targets via its output pathways. Most of the SCN's output is confined to the hypothalamus, and given the importance of the SCN in regulating physiology and behavior, the output pathways are surprisingly sparse and confined largely to the hypothalamus [75]. The SCN plays an important role in the regulation of sleep and appears to exert its effect on sleep primarily through projections to the subparaventricular zone and secondary connections to the dorsomedial hypothalamic nucleus which projects to brain regions involved in the regulation of sleep–wake behavior [76]. Lesions to the dorsomedial nucleus

eliminate rhythms in sleep, locomotor, feeding, and corticosteroid rhythms but not melatonin and body temperature [77].

The SCN also projects to the paraventricular hypothalamic nucleus in regulating melatonin (MT) synthesis. Regulation of the pineal gland is indirect [78]. The pathway leads to inhibition of MT synthesis during the day, when the SCN is active, and release from inhibition at night. As with many of the outputs from the SCN, the pathways are reciprocal. MT can inhibit SCN electrical activity via the MT1 receptor [78].

The SCN may also exert its action via diffusible signals. SCN transplantation experiments revealed that SCNs that were encapsulated in a polymer, preventing neural outgrowth, were capable of restoring locomotor rhythmicity in SCN lesioned hamsters [79]. Two diffusible mediators have been identified that may play key roles in the circadian regulation of physiology and behavior. Transforming growth factor (TGF- α) exhibits a circadian rhythm within the SCN and infusion of TGF- α into the third ventricle leads to inhibition of wheel running and sleep and body temperature rhythmicity [80]. A second diffusible factor, prokineticin 2, is a secreted protein that exhibits a circadian rhythm in the SCN with higher levels expressed during the subjective day. As with TGF- α , injection of prokineticin 2 into the brain results in inhibition of locomotor activity [81].

1.5 AGENTS THAT AFFECT PERIOD AND PHASE OF SCN

Many observations have demonstrated that the circadian pacemaker can be phase shifted and reset. In animals and humans these phenomena typically do not take place instantaneously but require from a few days to one week in order to completely shift the clock to a new phase. The speed is dependent on the direction of the shift and how large the shift is. As a general rule, phase delays occur more rapidly than phase advances.

Because SCN properties can easily be studied *in vitro*, there is now detailed knowledge about effects of phase-shifting agents on the SCN rhythm. One way to accurately determine the phase of the SCN rhythm is to record spontaneous electrical activity in SCN neurons, which is typically increased during the mid-subjective day as compared with subjective night [65, 82–84]. This technique has been widely used in many studies, and from these reports a complicated model of rhythm entrainment in mammals has slowly evolved, involving a number of pathways, neuromodulators, and transmitters. Light is the primary synchronizing signal to the SCN. Photoc information is conveyed principally to the ventrolateral (VL), also named “core,” region of the SCN by glutamatergic input via the retinohypothalamic tract (RHT), a group of axons within the optic nerve that originate from a restricted group of retinal ganglion cells and project directly to the VL SCN [85, 86]. Nonphotic input also reaches the VL SCN via neuropeptide Y (NPY) and γ -aminobutyric acid (GABA) in the geniculohypothalamic tract (GHT) and the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) and serotonin from the median raphe nucleus [87]. According to the current model of mammalian entrainment, activation of photoreceptors in the rodent retina causes glutamate release from SCN terminals of the RHT [88]. In retinorecipient SCN neurons glutamate release causes depolarization, Ca^{2+} influx, and Ca^{2+} -activated cAMP response binding element

(CREB)-dependent activation of clock genes [89–92]. Pituitary adenylate cyclase-activating polypeptide (PACAP) is colocalized with glutamate in RHT terminals [93] and may be involved in photic signal transduction [94–97]. PACAP potentiates glutamate-induced phase delays during the early subjective night and blocks glutamate-induced phase advances during the late night [95]. However, the effects appear to be complex, and the exact physiological role of PACAP is not yet certain [98]. In a diurnal rodent, the degus, it has been suggested that retinal innervation also includes the inhibitory transmitter GABA, which may indicate substantial differences in photic entrainment in diurnal versus nocturnal animals [99].

As described earlier, similar to the *Bulla* retinal clock, light phase delays the SCN in early subjective night and phase advances in late subjective night. In contrast, light stimuli during the subjective day have little effect [10]. The phase-shifting responses can be mimicked with depolarizing agents. For instance, glutamate, *N*-methyl-D-aspartate (NMDA), and nitric oxide (NO) generators cause a light-resembling phase response curve (PRC) in vitro [100] and optic nerve stimulation in the SCN in vitro generates a similar PRC as light [101]. Furthermore, NMDA activation causes lightlike behavioral phase shifts [102–104]. Several studies suggest that phase delays and advances of the SCN rhythm involve a number of intracellular cascades that take place at different circadian phases [105]. During the subjective day cAMP/protein kinase A (PKA) and PACAP phase advance the SCN, but these compounds have no effect during the subjective night [94, 106, 107]. However, phase shifts during the subjective night can be generated by glutamate (light), NMDA, NO, acetylcholine, and cyclic guanosine monophosphate (cGMP) [100, 108]. Adding another level of complexity, the effect of glutamate varies within the night period. In early subjective night glutamate stimulation induces Ca^{2+} -mediated intracellular Ca^{2+} release by ryanodine receptors that cause phase delays [109]. In late subjective night, on the other hand, it has been suggested that glutamate stimulation instead would activate the guanylyl cyclase (GC)/cGMP/protein kinase G (PKG) pathway and thereby cause phase advances [110].

Photic responses can be shaped by nonphotic inputs. For instance, the well-studied transmitters 5-HT and NPY phase shift the clock during the day and also modulate photic signaling [87]. The role of 5-HT is not clear, partly because of contradictory results in different species and experimental differences in vitro versus in vivo. Several studies, however, have demonstrated that 5-HT and its agonists cause robust phase shifts in SCN brain slices during the subjective day, and it has been suggested that the serotonergic pathway is involved in light entrainment to the SCN [111]. NPY reduces light-induced and light-like phase delays and advances in vivo and in vitro [87]. The pineal hormone melatonin is another mediator of light response at dusk and dawn and has been reported to gate sensitivity in the SCN via protein kinase C [112–115]. Melatonin is released during the dark period in both nocturnal and diurnal animals. Its exact function is not known; however, there is an ongoing debate of whether melatonin consolidates sleep and might be useful for therapeutic reasons to treat circadian rhythm sleep disorders as well as jet lag and disturbed rhythmicity due to shift work. The impact of melatonin levels has also been discussed regarding psychiatric diseases. The issue is certainly interesting since melatonin may have many implications in sleep and rhythmic disorders and a large number of studies examining melatonin release in patients with major depression (MD) and seasonal affective disorder (SAD) have been performed. However, these studies have reached different conclusions, and it is yet not clear if MD and SAD can

be correlated to altered concentrations of melatonin. In photoperiodic animals such as sheep and hamster melatonin has a more well-defined role and acts by coding the reproductive season [116].

1.6 CHRONOPHARMACOLOGY

Abrupt disturbances of circadian rhythms may cause insomnia, dysphoria, loss of appetite, gastrointestinal problems, fatigue, and difficulties concentrating, typical effects observed after rapid traveling across time zones, so-called jet lag [117]. Importantly, rhythmic dysregulation and desynchrony may also appear and play a major role in development of severe disease conditions, for instance affective disorders [118] and certain cancer forms [119]. The distress caused by circadian disruption is often correlated to increased levels of glucocorticoid secretion, which may in turn feed back negatively on the circadian entrainment system [120, 121]. The organization of time appears to have major impact in certain human diseases; for instance, asthma attacks, ulcer disease, hypertension, and coronary infarction clearly follow a circadian pattern of occurrence and/or severity that needs to be considered during treatment. This has resulted in the development of *chronopharmacology*, therapy based on the theory that most physiological functions follow a circadian rhythm and suggests the importance of using chronopharmacologically adjusted treatment in practical medicine [122]. Furthermore, the common prevalence of sleep disturbances associated with circadian dysfunctions in mood disorders emphasizes the necessity to develop new preventive and therapeutic strategies with chronobiological approaches. Drug efficacy for most compounds, including antipsychotics, antidepressants, benzodiazepines, and barbiturates, follows a circadian rhythm [123]. If biorhythms can be restored or strengthened by chronobiologically administered drugs or other treatments, the individual's life quality, and perhaps even life span, can be increased.

1.6.1 Affective Disorders and Lithium

During the last decade extensive research has been conducted regarding the circadian component in unipolar and bipolar disorders. Certain symptoms (daily variation in mood, early awakenings in the morning, seasonal relapses, altered rhythm in body temperature, increased concentration of melatonin, and disturbances in the circadian release of cortisol and thyrotropin) may be associated with a fundamental dysregulation of circadian rhythms, and it has been suggested that situations that result in an alteration of circadian rhythms can be correlated to an increased risk of relapse in bipolar patients [118, 124]. If the circadian system is altered in the disease, therapeutic substances should hypothetically also exert an effect on rhythmicity. In bipolar disorder the main drug of choice is lithium. Indeed, a number of experimental studies have demonstrated that lithium does have effects on circadian rhythms in animals and humans. These effects include lengthening of circadian period, phase shifts, reduction of circadian amplitude, and changes in light sensitivity and stabilization of rhythms. This literature has grown large, but a brief summary of a large portion of the main findings will be given here.

The fact that lithium may "slow down" and impact circadian rhythms was suggested already a few decades ago [125–127]. Seggie and colleagues performed

several studies examining the effect of lithium on circadian rhythmicity and found that lithium phase shifted behavior and the rhythmic release of melatonin and other hormones, although the direction of the phase shifts differed in some studies [128, 129]. Welsh and Moore-Ede [130] demonstrated that lithium significantly lengthened the circadian period, but not amplitude, of perch hopping in squirrel monkeys in a dose-dependent fashion. It should be mentioned that the primate study was performed in constant light, not darkness. Importantly, long-term constant light desynchronizes the circadian rhythm in mice and may therefore have major impact on studies of circadian rhythms [131]. In our laboratory we used the *Bulla* mollusk model to study the effect of chronic lithium chloride on the spontaneous firing rhythm in the *Bulla* clock neurons and found that the period was lengthened by lithium in a concentration-dependent fashion, an effect that was enhanced by light [132]. Furthermore, lithium pulses in combination with depolarizing K^+ pulses significantly phase delayed the pacemaker. Our group also showed that lithium similarly lengthens the period in cultured mouse SCN neurons [133], and recently it was reported that mouse locomotor activity rhythms also are lengthened by lithium [134]. LeSauter and Silver [135] showed that lithium also lengthened the circadian activity period in lesioned hamsters with SCN grafts. Hafen and Wollnik [136] studied the effect of lithium in three different rat strains that showed altered circadian activity patterns. The investigators found that lithium caused a period lengthening as well as a decrease in amplitude and general reduction of the activity rhythm. The authors therefore discussed the possibility that decreased activity would feed back on the circadian period and the effect of lithium would therefore not be primary. The authors' findings did not support this theory because they could only find a significant correlation between activity level and period lengthening in one of the rat strains. The authors therefore concluded that their results strengthened the hypothesis that lithium acts pharmacologically on the pacemaker system and that the observed effects were not secondary to a feedback from the reduced overall activity level. However, a secondary effect due to negative side effects and activity feedback on the pacemaker could not be completely excluded. Klemfuss and Kripke [137] found that lithium stabilizes circadian rhythms in hamster. Similar to McEachron et al. [125], Subramanian et al. [138] studied lithium effects on biochemical parameters in Wistar rats. Chronic lithium treatment delayed rhythms in glucose, Ca^{2+} , and K^+ but advanced the rhythms in malondialdehyde, cholesterol, and lactic acid. McEachron et al. [125] also found both delays and advances of biochemical rhythms, where 8 of 11 parameters were delayed and 3 of 11 were advanced. As an explanation the authors suggested that biochemical rhythms are linked with two different oscillators that are differently affected by lithium. In chick pineal gland, lithium produced two peaks of melatonin secretion in which one of them was delayed as compared with the single control peak [138a], and lithium has a lengthening effect of the period in *Drosophila* maintained in constant darkness [139]. Although secondary effects due to side effects and activity feedback cannot be completely excluded, the studies described so far strongly suggest that lithium in most species lengthens and may stabilize circadian period in experimental animals, possibly by influencing glycogen synthase kinase-3 in the master pacemaker [134, 139]. However, a slightly different conclusion was reached in a paper by Williams and Jope [140]. The authors studied the transcription factor activator protein 1 (AP-1) DNA binding activity in rat cerebral cortex stimulated by a cholinergic agonist and

found that stimulated AP-1 binding was influenced by a circadian rhythm. When chronic, but not acute, lithium administration was administered, however, this circadian component was abolished. Although this study did not support the stabilizing effect of lithium on circadian rhythms, it was not determined whether the effect was caused by a direct effect of lithium on the circadian rhythm or if the effect was indirect via inhibition of signaling immediate early gene pathways involved in AP-1 activity, which in turn could influence the circadian clock.

In humans, lithium also appears to have a lengthening effect of the circadian period in both healthy individuals and patients with bipolar disorder and major depression and may even work as a prophylactic [141, 142]. It has also been reported that lithium enhances chronobiological treatments such as sleep phase advances in bipolar patients [143].

The mechanism of lithium may involve modulation of intracellular and extracellular ion concentrations. Lithium depolarizes neurons and modulates the concentration of potassium in the brain, possibly by blocking potassium channels and inhibiting the Na^+/K^+ adenosine triphosphatase (ATPase) [144–146]. Interestingly, recent studies strongly suggest that ionic membrane conductances such as K^+ and Ca^{2+} may be involved in the intracellular circadian machinery [71, 147–149]. Lithium may therefore impact the circadian clock directly by modulating membrane potential and K^+ flux.

In summary, a large pool of data suggests that lithium does affect circadian rhythms to some extent, possibly by influencing the circadian clock and lengthening and stabilizing the circadian period. However, this statement still needs to be acknowledged with some caution because the nature of the circadian effects is not fully consistent between studies.

1.6.2 Antidepressants and Effects on Sleep

Many antidepressants affect the sleep architecture. Both negative and positive effects on sleep structure have been reported [150]. Whether these effects are circadian or impact the homeostatic sleep circuits has not been determined. Nevertheless, the circadian system and the sleep circuits are interacting and drugs may impact both systems simultaneously. In a study performed in rats, the selective serotonin reuptake inhibitor (SSRI) zimeldine initially increased wakefulness, followed by an increase in slow wave sleep (SWS) stage 2. Rapid-eye-movement (REM) sleep was abolished after zimeldine treatment. The authors also administered L-tryptophan, which decreased waking and increased SWS when administered in late, but not early, light phase, clearly demonstrating the impact of circadian drug sensitivity to L-tryptophan [151]. The drug fluoxetine had similar effects in Syrian hamsters in which REM sleep was significantly decreased and non-REM sleep was increased [152]. In addition, the drug decreased brain temperature. A partial tolerance to the reduction of REM sleep, but not decreased hypothalamic temperature, was developed during chronic administration of the drug. In humans, Silvestri et al. [153] administered the SSRIs paroxetine and fluvoxamine to healthy subjects and performed electroencephalographic (EEG) recordings. Both drugs disrupted the sleep pattern, paroxetine more so than fluvoxamine, and paroxetine-induced sleep disruption that persisted into the withdrawal phase. Sleep disruption and decreased sleep quality caused by another SSRI, fluoxetine, in depressed children was also demonstrated by Armitage et al. [154].

However, in contrast to earlier studies in adult subjects investigating fluoxetine's effect on sleep [154–156], Armitage et al. did not find that fluoxetine reduced REM sleep in children [154]. Nefazodone, on the other hand, had an improving effect on sleep quality [154].

A different result with paroxetine was obtained by Schlösser et al. [157]. In their subjects, paroxetine administration did not alter sleep efficiency, total sleep time, sleep onset latency, number of awakenings, and awake during sleep period times. However, in accordance with Silvestri et al. [153], paroxetine medication affected REM sleep (prolonged REM latency and a decrease in the number of REM phases). The same group investigated the effect on hormones at night and profiles were obtained for prolactin, growth hormone (GH), cortisol, corticotropin (ACTH), luteinizing hormone (LH), testosterone, and melatonin [158]. None of the hormonal profiles were significantly altered by paroxetine; however, fluoxetine hydrochloride increased the circadian amplitude in prolactin release in postmenopausal women [159]. Differences in the experimental protocols may explain the contradictory results regarding sleep architecture. Different doses of the drug (20 vs. 30 mg/day) were used, but more significantly, the time of administration differed between the studies. Silvestri et al. [153] administered the drug morning and evening whereas Schlösser et al. [158] gave the drug only in the morning to the subjects. Because the serotonin levels are normally high during the subjective day, the morning-only protocol may have produced less side effects due to an already increased concentration of 5-HT and therefore also did not affect the sleep pattern significantly. Not in accordance with this hypothesis is a study by Saletu et al. [158], who compared SSRI administration given in the morning versus the evening. Total sleep time and sleep efficiency deteriorated under both morning and evening dosing and nocturnal wake time and sleep stage 1 increased. However, a delayed sleep onset and REM latency was lengthened only after the morning intake.

Whether differences in the experimental protocols explain the opposite results or not, these results clearly demonstrate the importance of drug concentration and timing of drug administration not only in pharmacological therapy but also in experimental studies in order to compare obtained results between different drugs and studies.

1.6.3 Antidepressants and Circadian Rhythms

Because of the unclear role of 5-HT in the circadian system it is also difficult to interpret effects of SSRIs on circadian rhythms, and studies have reached different conclusions. However, there is no doubt that antidepressants exert major impacts on circadian rhythms, including change of period, phase, and entrainment. A few of them will be discussed here. Many studies have suggested that 5-HT modulates photic responses in the SCN [160]. Certain 5-HT-enhancing antidepressants attenuate phase shifts [161]. However, a different conclusion was reached by Moriya et al. [162], who found that 5-{3-[(25)-1,4-benzodioxan-2-ylmethyl] amino} propoxy}-1,3-benzodioxole HCl (MKC-242), a selective 5-HT (1A) receptor agonist, accelerated the reentrainment of hamster wheel-running rhythms after 8-h phase shifts and produced larger phase advances of the wheel-running rhythm produced by light pulses. Furthermore, the authors found that MKC-242 increased light-induced *Per1* and *Per2* mRNA expression in the mouse SCN and suggested this as a molecular

mechanism underlying the potentiated phase shifts [163]. However, a direct link between increased expression of *Per* and magnitude of phase shifts remains to be clarified, although it has been demonstrated that light exposure causes induction of *Per1* [164] and photic phase delays can be inhibited by *Per1* and *Per2* antisense oligonucleotides [165, 166]. The drugs 8-hydroxydipropylaminotetralin, paroxetine, buspirone, and diazepam did not have any significant effects on *Per* expression [163].

A study of circadian wheel-running activity after antidepressant administration was performed in rats [167]. Wheel-running activity rhythms were recorded in three inbred strains of laboratory rats in constant darkness, among which two of these strains showed abnormal multimodal circadian activity patterns. Chronic treatment with moclobemide, a reversible and selective monoamine oxidase (MAO) type A inhibitor, and desipramine, a preferential norepinephrine reuptake inhibitor, but not the SSRIs clomipramine and fluoxetine administered ad lib, consistently increased the circadian amplitude of the activity rhythm, and the abnormal bimodal activity pattern in one of the strains became unimodal. Furthermore, the free-running period was shortened by moclobemide and desipramine, an effect that persisted for a long time after treatment (aftereffects). Fluoxetine, however, which did not exert any effect in this study, suppressed food intake in a dose-dependent fashion during early night but not during mid and late night [168]. Although the studies measuring locomotor activity and food intake are not directly comparable, they demonstrated inconsistent results of fluoxetine on different kinds of rodent behavior. Differences in the experimental protocol such as time of drug administration may be one explanation. Another compound, the α adrenoceptor agonist clonidine, shortened the period and amplitude of circadian activity rhythms in constant conditions [169].

The effect on circadian cortisol release has also been under investigation because hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis is associated with major depression and may also involve abnormal circadian rhythmicity of cortisol levels [170]. Linkowski et al. [171] found that electroconvulsive therapy or amitriptyline restored hypersecretion of cortisol to control concentrations and the timing of the cortisol nadir (earlier in depressed patients before treatment) was normalized. Steiger et al. [172] reported that cortisol levels were returned to normal after treatment with tricyclic antidepressants (amitriptyline and imipramine) but secretion of growth hormone and altered EEG pattern remained disturbed in depressed subjects. In contrast, Rota et al. [173] found a positive effect of fluvoxamine and amitriptyline on the abnormal cortisol rhythm in patients with major depressive disorder, but this effect was not significant. From the report it is not clear when the drugs were administered. However, the lack of effect may not be related only to circadian parameters but may be due to nonresponsiveness caused by an activated HPA axis [174]. In rats Reul et al. [175] found that amitriptyline attenuated basal cortisol levels and Gomez et al. [176] demonstrated that phenelzine and imipramine altered the normal circadian pattern of corticosterone; however the circadian amplitude was reduced by the treatment, not enhanced. The experimental protocols differed in terms of drug administration; Reul et al. [175] administered antidepressants in the drinking water whereas Gomez and collaborators [176] administered once a day in the morning by injection.

The discrepancies between the above-discussed studies describe a large complexity of different antidepressant drugs and their effects on sleep and circadian rhythms. Certain antidepressants may themselves cause circadian rhythm sleep disorders,

manifested as sleep disturbances, day somnolence, and fatigue. These symptoms are commonly treated with benzodiazepines or other sleep-promoting substances. For instance, in one study by Hermesh and co-workers [177] fluvoxamine but not the other SSRIs fluoxetine and clomipramine caused circadian rhythm sleep disorder. From these studies it is impossible to draw one specific conclusion of how antidepressants, as one general group of compounds, affect the circadian system. It is clear, though, that the time of administration of several antidepressant drugs appears to be important in terms of drug efficacy and side effects such as sleep disorganization, which in turn suggests that many of these compounds may be powerful *zeitgebers* (“timegivers”) acting on the circadian pacemaker via the neurotransmitter systems in the brain. The impact on phase by these substances may be enhanced by classical *zeitgebers* such as light; for instance, light therapy given in the morning (giving a 2-h phase advance in each individual) during two weeks in combination with citalopram significantly improved the effect of the antidepressant drug in patients with MD and bipolar disorder compared to placebo [178]. Furthermore light therapy is an effective treatment in SAD [179]. Whatever the underlying mechanism of light therapy is, these and other reports again emphasize the importance of strengthening the circadian system as a powerful tool to improve neuropharmacological treatments. Chronobiological treatments and life-style strategies may also have a preventive and prophylactic effect on the reoccurrence of psychiatric disorders [180].

1.6.4 Benzodiazepines

It was discovered early that benzodiazepines could phase shift circadian rhythms in rodents, primates, and humans [181]. These effects may be mediated by the serotonergic system [182, 183]. It has also been suggested that triazolam facilitates phase shifts and would therefore help in adjusting to new time environments and attenuate jet lag and shift work effects [184]. However, facilitation of reentrainment by triazolam could not be demonstrated in squirrel monkeys [185]. Again, the timing of drug administration is crucial for reaching maximum beneficial effects [184] and benzodiazepines may cause both advances and delays depending on when during the circadian cycle they are administered. It has also been demonstrated that locomotor activity may be required for benzodiazepine-induced phase shifting in hamsters. Van Reeth and Turek [181] found that when hamsters were immobilized, triazolam- (which does not have a sedative effect in these animals) induced phase advances and delays were inhibited. In one study of healthy subjects benzodiazepines reduced the melatonin levels and altered the rhythmic profile of the hormonal release [186]. The mechanisms may involve GABA_A receptors because it was found that flunitrazepam increases the duration of spontaneous GABAergic postsynaptic currents and potentiates the amplitude of GABA evoked currents in rat brain slices of the SCN [187]. The effect of diazepam and triazolam on clock gene expression was investigated in the mouse cerebellum. The benzodiazepines acutely reduced *Per1* messenger RNA (mRNA) level when injected at *zeitgeber* time (ZT; time in hours after light goes on) 4 and 16 but the effect was transient [188]. Vansteensel et al. [189] pointed out another level of complexity of the phase-shifting effects of non photic stimuli like benzodiazepines: Midazolam and the opioid receptor agonist fentanyl induced phase advances of *activity onset* but not *activity offset* when given

mid- to late subjective day in hamsters. Finally, Subramanian and Subbaraj [190] found that when diazepam was given in the drinking water to mice, the free-running period was either shortened or lengthened and phase shifts introduced during the end of the circadian cycle were attenuated in the treated animals. Nevertheless, benzodiazepines clearly can shift circadian rhythms in humans and experimental animals. If used wisely, these compounds may be helpful in overcoming negative phase-shifting effects such as jet lag but also have negative effects if used at the “wrong” time. Interestingly, in an early study by Okawa et al. [191], a blind child was entrained for several years by administration of nitrazepam. More specifically designed studies on benzodiazepine effects on phase shifting in real life, however, are necessary in order to determine exactly how these drugs should be used in this context.

1.6.5 Inflammatory Molecules

Disturbances in biological rhythms, especially the sleep–wake cycle, not only are associated with psychiatric mood disorders but also involve numerous physical and cognitive disorders. Such disturbances may be brought about by infectious diseases, autoimmune disorders, malignancies, or aging. In these conditions so-called *cytokines* are released for intercellular communication to activate or modify immune cell responses as well as cellular proliferation, maturation, and survival. There is now accumulating evidence that cytokines can also operate at the tissue or systems level in influencing the mammalian central nervous system. A number of cytokines have been ascribed somnogenic properties [192], but recent observations suggest that cytokines also are involved in mediating a number of physiological impairments via their effect on the circadian timing system. Because of their great impact during common infections and a number of inflammatory conditions, we will review what is known so far about cytokines and circadian rhythms.

Cytokines are small secreted proteins that exhibit autocrine or paracrine actions to regulate the intensity and duration of immune responses. They stimulate the activation, proliferation, or differentiation of cells involved in the defense against microbial invasion of an organism. There are proinflammatory cytokines, for instance interleukin (IL) 1, tumor necrosis factor (TNF) α , and interferon (IFN) γ that amplify inflammatory reactions, and anti-inflammatory cytokines, such as IL-4, IL-6, and IL-10 that are involved in the reduction of proinflammatory reactions [193]. Although first identified in the immune system, cytokines can be produced by glial cells and affect functions in the brain.

1.6.6 Cytokines and Disturbances in Circadian Sleep–Wake Rhythms

Cytokines and microbial products released during an infection and/or immune activation cause physiological and psychological effects that are collectively termed *sickness behavior*, characterized by anorexia, depressive symptoms, muscle aches, somnolence, and lethargy [194]. Proinflammatory cytokines, in particular IL-1 and TNF- α , are well known for being somnogenic and microbial products such as lipopolysaccharide (LPS) exert their effects in this respect by being potent inducers of these cytokines. Increased cytokine release occurs during aging and in age-related neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease.

During these conditions nocturnal disruptions of sleep, manifested as agitation during nighttime, and increased number of sleep episodes during the day are common [195–198]. Patients with cancer and multiple sclerosis (MS), diseases that also are associated with elevated cytokine release, may also show severe disturbances in the sleep–wake architecture and altered rest–activity patterns [199–201]. Patients with cancer and MS often suffer from MD [202–204] that could contribute to the sleep disturbances. In developing countries in sub-Saharan Africa the infectious disorder sleeping sickness [human African trypanosomiasis (HAT)] is reemerging. The disease is caused by infection with the extracellular parasite *Trypanosoma brucei* that results in immune activation and early release of the proinflammatory cytokines IFN- γ and TNF- α [205]. Severe fragmentation of the sleep–wake rhythm characterizes the disease, which increases progressively during the course of disease [206, 207]. Similar disruptions in the sleep patterns occur in rodents experimentally infected with the parasites, and trypanosome-infected animals therefore provide a model to study pathogenic mechanisms underlying disturbances in sleep pattern and rhythms [208].

In patients afflicted with these conditions, but also in subpopulations of aging individuals, increased concentrations of cytokines have been found in plasma, cerebrospinal fluid (CSF) and/or brain. Considering the proinflammatory cytokines, patients with Alzheimer's disease exhibit elevated concentrations of IL-1, IL-6, and TNF- α [209, 210], and in a rodent model of this disease increased levels of IL-1, TNF- α , and IFN- γ have been found [211]. Increased levels of circulating IL-1, IL-6, TNF- α , and IFN- γ have been associated with MD [212, 213]. Similarly, increased concentrations of IL-1, IL-6, TNF- α , and IFN- γ have been found in subpopulations of elderly humans as well as in old mice and rats [214–219]. In sleeping sickness patients, and in experimental rodent models of African trypanosomiasis, there is marked increase of IL-1, IL-2, IL-4, IL-10, TNF- α , and IFN- γ in the central nervous system [220, 221]. A positive correlation between plasma levels of IFN- γ and the severity of the disease has been demonstrated by Radomski et al. [222]. In spite of the fact that somnogenic proinflammatory cytokines such as IL-1 and TNF- α are released in both aged individuals and patients affected with these diseases, with the exception of certain forms of MD, there is usually no hypersomnia. Rather, a common feature appears to be a disruption of the sleep–wake pattern, pointing to an alteration not only in the sleep circuits but also in the circadian system.

Direct evidence for linking cytokines to disturbances in sleep and perhaps circadian rhythms in humans has been obtained from studies on patients with MS and certain forms of cancer undergoing intensive immunotherapies. IFN- γ , IL-2, and TNF- α can cause MD that may include sleep and circadian rhythm disturbances [124, 223, 224]. Conversely, it has been suggested that certain patients suffering from depression may have a “chronic inflammation” and reducing inflammation could offer a way to treat such disorders [212].

Recent experimental data investigating more directly effects of cytokines on circadian parameters suggest that some of these molecules may be potent modulators of circadian clock functions. In the rat brain, the receptor for IFN- γ is located in the ventrolateral part of the SCN and in areas such as the piriform and entorhinal cortex, midline thalamus, medial hypothalamus, and tubero-mammillary nuclei [225]. When rats were entrained in a 12:12 h LD cycle, but not in free-running conditions, the receptor expression in the SCN was low during the subjective day and increased

during the early subjective night, with the highest expression at ZT 15 [226, 227]. Other data also point out an influence of IFN- γ on the circadian timing system. For example, IFN- γ administered subcutaneously in mice at ZT 12, but not at ZT 0, caused a marked reduction of the mRNA levels of *per* in the SCN [228] and intracerebroventricular injections of IFN- γ alters locomotor activity in golden hamsters [229]. IFN- γ operates in synergy with TNF- α [230], which in fact was first isolated from rabbits infected with trypanosomes under the name of cachectin. IFN- γ and TNF- α in combination with bacterial LPS blunted the spontaneous firing activity and reduced spontaneous excitatory postsynaptic activity in slices of the SCN [231]. Furthermore, we have recently found that IFN- γ decreases GABAergic synaptic activity and modulates decreases impulse pattern in dispersed SCN neurons [232]. Bacterial LPS phase shifts activity rhythms in mice during early subjective night, similar to photic signals [233]. Another proinflammatory cytokine, IFN- α , has also been demonstrated to have a major impact on clock functions. In a study by Koyanagi and Ohdo [234] IFN- α blunted rhythmic expression of CLOCK and BMAL1, rhythmic behavioral activity, and rhythmic release of vasopressin. Taken together, the recently accumulating number of studies examining cytokine effects on brain function strongly suggest that cytokines alter not only sleep functions but also SCN physiology and circadian output both in vitro and in vivo.

1.7 CONCLUDING REMARKS

Circadian rhythms are fundamental processes in most organisms which in humans appear to have major impact on physiological processes, life quality, and well-being. This has become more apparent during the last few decades when development of a high-technology and high-efficiency society has resulted in more artificial life environments, such as shift work and frequent business traveling across time zones, as well as increased stress levels resulting in sleep and rhythm dysfunctions. In addition, more advanced pharmacological therapies now available in the clinics have pushed the development of sophisticated therapeutics to involve chronopharmacological strategies as powerful tools to optimize drug effects and minimize toxic side effects of compounds used for a number of diseases, including psychiatric disorders, infectious diseases, inflammatory conditions, autoimmune disorders, and neurodegenerative diseases. With the increasing average life span of humans in Western society, circadian rhythm disorders associated with aging have also attracted more attention and focus for chronobiologists. In other words, circadian rhythm disorders are phenomena belonging to modern society and the life style that is associated with modern environments.

The master clock in the hypothalamus is a remarkable structure with a well-defined biological function: the generation and maintenance of an internal timing program in the body which can be severely disrupted by neurotransmitters, hormones, pharmacological compounds, and immune system mediators, resulting in substantial negative effects on physiological functions. Although not reviewed here, commonly used drugs as caffeine [235], nicotine [236], and alcohol [237] may also influence circadian parameters and change period, amplitude, and entraining properties. Rhythmic disturbances may play a role in linking certain conditions to others; for instance, alcohol intake may alter rhythms and alcoholism is commonly associated with major

depression, which in turn is characterized by sleep and rhythm alterations. The circadian aspect may in this context have an unforeseen role in that strengthening the timing machinery could improve the outcome of treatment of these disorders and others. Finally, the importance of maintaining the circadian program as intact as possible has also become evident in development of tumors [238–240], and circadian clock function is important for the cell cycle, DNA damage response, and tumor suppression in vivo [241]. Studies of circadian rhythms and circadian clock functions have entered a new era of research and reached a higher level of importance, perhaps even greater than the original pioneers in the field ever could imagine.

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2

MELATONIN RECEPTORS IN CENTRAL NERVOUS SYSTEM

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2.1	Introduction	37
2.2	Historical Perspective	38
2.3	Melatonin Production	40
2.4	Melatonin Receptors	41
2.4.1	Molecular Structure	41
2.4.2	Molecular Pharmacology	42
2.4.3	Signaling	44
2.5	The Suprachiasmatic Nucleus	46
2.5.1	Circadian Inputs and Outputs	46
2.5.2	Melatonin Receptors in SCN: Localization, Signaling, and Function	47
2.5.3	Melatonin Receptors and Clock Genes	50
2.5.4	Regulation	51
2.5.4.1	Desensitization	52
2.5.4.2	Supersensitization	53
2.6	Melatonin Modulation of Circadian Rhythms of Behavior	54
2.7	Melatonin Receptors as Therapeutic Targets	57
2.7.1	Sleep	57
2.7.2	Circadian Rhythms	58
2.7.3	Depression	58
	Acknowledgments	61
	References	61

2.1 INTRODUCTION

The hormone of darkness melatonin (*N*-acetyl-5-methoxytryptamine) is released by the pineal gland in a circadian fashion defining the length of the night and relaying photoperiodic information to the organism [1]. Pineal melatonin provides circadian

and seasonal timing cues for the regulation of prolactin secretion, color coat, and reproduction in seasonal breeders [2–7]. Furthermore endogenous melatonin promotes sleep [8], fine tunes phase position and amplitude of circadian rhythms [9], and participates in the regulation of neuroimmune function [10, 11] and inhibition of cancer tumor cell growth [12]. Locally released melatonin modulates dopaminergic transmission in retina and participates in the transmission of visual and circadian information to the brain [13, 14]. Exogenous melatonin through activation of melatonin receptors in the central nervous system modulates a myriad of physiological functions, including sleep [15–18], circadian rhythms [18–20], and visual [21], cerebrovascular [22], neuroendocrine [23, 24], and neuroimmune [11] functions. This review will describe the cellular and signaling mechanisms by which activation of MT₁ and MT₂ melatonin receptors in the central nervous system could be involved in the treatment of insomnia, circadian rhythm disturbances, and neuropsychiatric disorders.

2.2 HISTORICAL PERSPECTIVE

Melatonin is synthesized in the pineal gland of all vertebrate species, including humans. The biological activity of melatonin dates back to 1917 when McCord and Allan (1917) discovered the blanching effects of bovine pineal extracts on tadpole skin. This bioassay was used by Lerner et al. (1959) [25] to isolate melatonin from pineal extracts and to unravel its chemical structure. The property of melatonin to aggregate pigment granules (melanosomes) in amphibian dermal melanophores was critical to demonstrate for the first time the presence of functional melatonin receptors, the structure–activity relationship of melatonin-like ligands, the coupling of melatonin receptors to pertussis toxin–sensitive G proteins, and the cloning of the first complementary DNA (cDNA) encoding a seven-transmembrane domain melatonin receptor (Mel1c) [26–28].

Aaron Lerner discovered melatonin and was the first to report its ability to induce sleepiness during the day in humans [29, 30]. This observation was followed by reports demonstrating a direct effect of melatonin on sleep processes in humans using physiological (0.3-mg) to suprapharmacological (85-mg) doses [17, 31–35]. The role of melatonin in the treatment of circadian rhythm disorders followed the publications by Redman and colleagues [36] demonstrating melatonin-mediated entrainment of free-running activity rhythms of rats kept in constant dark and by Murakami et al. [37] reporting that melatonin implants near the suprachiasmatic nucleus (SCN) accelerated reentrainment of cortisol rhythms. These pioneer studies on melatonin-induced sleep and phase shift of circadian rhythms prompted the search for melatonin receptor ligands to treat circadian and not-circadian sleep disturbances in humans (for a review see [17, 18]).

Melatonin receptor activation appears to modulate serotonin (5-HT) receptor–mediated signaling in the brain. Small doses of melatonin delivered into the rat nucleus accumbens decrease locomotor activity and rearing and increase grooming and sniffing behavior [38]. These behavioral effects are counteracted by 5-HT agonists and tricyclic antidepressants but not by dopamine antagonists, suggesting an interaction between 5-HT and melatonin receptor–mediated signaling in the nucleus accumbens [38]. Melatonin, as demonstrated for 5-HT_{1A} agonists and

antidepressants, inhibited 5-HT_{2A} receptor-mediated headshakes and stimulation of phosphoinositide hydrolysis in rat cortical slices through a receptor-mediated mechanism [39]. Melatonin administered in vivo reduced 5-HT_{2A} receptor agonist-induced increase in phosphoinositide hydrolysis without affecting receptor density, further supporting the interaction of the melatonin and 5-HT systems. Melatonin also modulates the sensitivity of 5-HT₂ receptor-mediated sleep responses in the rat [40]. More recently, the discovery that agomelatine, an agonist for MT₁ and MT₂ receptors and antagonist at 5-HT_{2C} and 5-HT_{2B} receptors, is an effective antidepressant has revived the interest in the potential interactions between heterologous regulation of 5-HT and melatonin receptor signaling [41].

Concurrent biochemical, molecular, and pharmacological studies localized and established the function of melatonin receptors in the mammalian central nervous system. The discovery of functional melatonin receptors inhibiting dopamine release in rabbit retina together with the report of the structure-activity relationships for melatonin ligands [13, 42] led to the design and synthesis of the first competitive melatonin receptor antagonist, luzindole (*luz*: Spanish for “light”; *indole*: basic chemical structure of melatonin). Luzindole (2-benzyl-*N*-acetyltryptamine) was designed to mimic light signals by blocking the effects of melatonin, the hormone of darkness.

The development of 2-[¹²⁵I]iodomelatonin as a high-affinity radioligand [43–45] allowed the localization and pharmacological characterization of melatonin receptors in mammalian retina, brain, and peripheral tissues as well as the cloning of the first nonmammalian melatonin receptor (for a review see [46–49]). The cDNA encoding the *Mel1c* melatonin receptor was cloned by expression of purified messenger RNA (mRNA) from *Xenopus laevis* melanophores into mammalian cells in culture following detection by receptor autoradiography with 2-[¹²⁵I]iodomelatonin [28]. This landmark discovery led to the cloning of the mammalian melatonin receptors *Mel1a* and *Mel1b*, belonging to a novel subfamily of seven-transmembrane-domain G-protein-coupled receptors (GPCRs) [50, 51]. These melatonin receptors, currently referred to as MT₁ and MT₂, respectively, exhibit distinct molecular and pharmacological characteristics and are localized to different chromosomes (MT₁, 4q35.1; MT₂, 11q21–q22) [19, 52–54]. The pharmacological characterization of the mammalian MT₁ and MT₂ melatonin receptors has advanced the discovery of selective and specific ligands for these receptors [52, 55–59]. The introduction of two prototype competitive melatonin receptor antagonists luzindole and 4P-PDOT (4-phenyl-2-acetamido tetraline) [52, 60] and of mice with genetic deletion of MT₁ and/or MT₂ melatonin receptors [61, 62] allowed the functional characterization of melatonin-mediated responses in native tissues. The mammalian MT₁ and/or MT₂ G-protein-coupled melatonin receptors are viewed as potential targets for the development of melatonin receptor ligands to treat disorders involving alterations in sleep and in the phase of circadian rhythms as those observed in subjects with depression, blindness, and delay sleep phase syndrome or following rapid changes in the light–dark cycle as occur during jet travel and shift work [18, 63]. The role of melatonin receptors in the regulation of physiological functions outside the central nervous system has been recently reviewed elsewhere [22, 49].

The putative MT₃ melatonin site binds 2-[¹²⁵I]iodomelatonin with nanomolar affinity and displays a distinct pharmacological profile from other melatonin

receptors (i.e., affinity profile for MT₃ is *N*-acetylserotonin ≥ melatonin » serotonin and for MT₁ and MT₂ is melatonin » *N*-acetylserotonin » serotonin) [48, 53, 64]. 5-MCA-NAT (5-methoxycarbonylamino-*N*-acetyltryptamine), prazosin, and *N*-acetyltryptamine are selective ligands for the MT₃ site [64]. The MT₃ melatonin binding site was originally thought to be a GPCR [65, 66]. However, a protein isolated from hamster kidney and identified as quinone reductase II binds 2-[¹²⁵I]iodomelatonin and the selective MT₃ ligand 2-[¹²⁵I]-MCA-NAT with the same pharmacological profile described for the MT₃ site in both hamster brain and kidney membranes [67, 68]. The MT₃ binding site is absent in brain and kidney membranes from mice with genetic deletion of quinone reductase II [68, 69]. The complete identity of MT₃ membrane binding sites with quinone reductase II as well as the physiological relevance of these sites remains to be determined.

2.3 MELATONIN PRODUCTION

In mammals, melatonin is synthesized primarily by the pineal gland and the retina and is released in a circadian fashion with high levels during the night. The synthesis of melatonin in the pineal gland is regulated by the master biological clock, the SCN of the hypothalamus, via multisynaptic neuronal pathways and/or neurochemical transmission. Signals of dark and light are transmitted from the retina to the SCN via the retinohypothalamic tract (Fig. 2.1a). The SCN regulates the circadian production of melatonin in the pineal gland by transmitting neuronal signals through the paraventricular nucleus (PVN) of the hypothalamus, the intermediolateral (IM) cell column, and the superior cervical ganglia (SCG) (Fig. 2.1; for a review see [20, 70, 71] (Fig. 2.1a). A clock mechanism within the retina itself drives the melatonin rhythm in this tissue [14, 20]. The circadian clocks in the SCN and retina are entrained to a 24-h period by environmental light, which is sensed by the retina and is conveyed to the SCN via the retinohypothalamic tract.

Melatonin is synthesized from 5-HT through two enzymatic steps (Fig. 2.1b). First, the rate-limiting enzyme serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase; AA-NAT) acetylates 5-HT to yield *N*-acetylserotonin (NAS). The second step involves transfer of a methyl group from *S*-adenosylmethionine to the 5-hydroxy group of *N*-acetylserotonin via the enzyme hydroxyindole-*O*-methyl transferase (HIOMT) [70, 72] (Fig. 2.1b). Interestingly, 5-HT levels in the pineal gland follow a triphasic pattern with constant levels during the day, a sharp increase at lights off, and a concomitant decline as the levels of melatonin increase [73]. Transcriptional and posttranscriptional mechanisms regulate the daily rhythms of AA-NAT [20, 70, 74–76] and HIOMT [77] activity. Circadian melatonin secretion from the pineal gland shows the acrophase during the dark period and a nadir during the light phase in both diurnal and nocturnal species [18]. Light exposure during the dark phase suppresses melatonin production [78]. In animals as well as in humans maintained under the adequate photoperiod, the duration of melatonin secretion is longer during the winter nights and shorter during the summer nights, which may contribute to differential seasonal responses mediated by endogenous melatonin [78]. Low levels of AA-NAT and HIOMT have been also detected in brain and peripheral tissues (spinal cord, raphe nucleus, striatum, gut, testis), suggesting melatonin synthesis in these tissues [79].

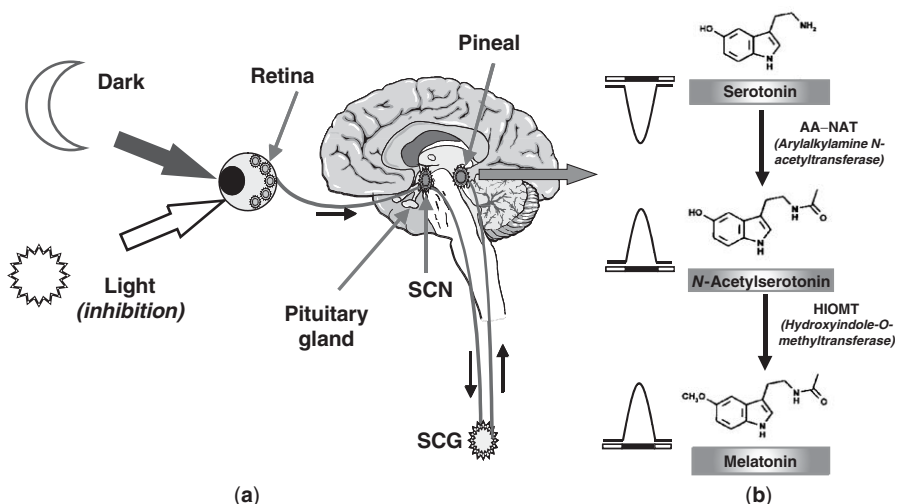


Figure 2.1 Regulation of melatonin synthesis. Melatonin is synthesized in the retina and in the pineal gland. In the pineal gland, melatonin synthesis follows a rhythm driven by the SCN, the master biological clock, following a multisynaptic path, including, among others, the SCG. Norepinephrine released from postganglionic fibers activates α_1 and β_1 adrenoreceptors in the pinealocyte, leading to increases in second messengers [i.e., cyclic adenosine monophosphate (cAMP) and inositol trisphosphate] and in the activity of AA-NAT, the rate-limiting step in melatonin synthesis. The system is dramatically inhibited by light, the external cue that allows entrainment to the environmental light–dark cycle. The photic signal received by the retina is transmitted to the SCN via the retinohypothalamic tract, which originates in a subset of retinal ganglion cells. The pineal melatonin thus serves as the internal signal that relays day length, allowing regulation of seasonal changes in reproductive physiology and behavior. The pars tuberalis of the pituitary gland interprets this rhythmic signal and generates a precise cycle of expression of circadian genes. Melatonin synthesis in the photoreceptors of the retina follows a similar daily rhythm that is generated by local oscillators. (Reprinted from [71] with permission from Elsevier.)

2.4 MELATONIN RECEPTORS

2.4.1 Molecular Structure

In mammals, melatonin activates at least two high-affinity G-protein-coupled membrane receptors—the MT_1 and the MT_2 —that mediate a myriad of functional responses [19, 47]. The best-characterized signaling pathways for the mammalian MT_1 and MT_2 melatonin receptors is through coupling to heterotrimeric G proteins ($G\alpha\beta\gamma$) and dissociation into $G\alpha$ and $G\beta\gamma$ dimers that interact with effector molecules involved in the transmission of cell signaling. The MT_1 and MT_2 melatonin receptors show distinct molecular structure, pharmacological profile, and chromosomal localization [52, 54, 80]. The molecular structure consists of seven transmembrane (TM) helices (I–VII) linked by three alternating intracellular and extracellular loops. Melatonin receptors are a distinct group within the family of GPCRs having an NRY motif rather than a DRY or ERY motif that is present in intracellular loop II of all other GPCRs [50]. Mutation of the Asn124 in the NRY motif of the MT_1 melatonin receptor indicated that this amino acid regulates cAMP signaling, calcium channel activity, and receptor trafficking [81]. Furthermore, in the

MT₁ melatonin receptor the conserved cysteines C127 and C130, localized at the end of transmembrane domain III, are needed for normal G-protein activation and receptor trafficking [82]. Biochemical studies revealed sulfhydryl groups to be important for melatonin binding [83, 84]. A disulfide bond between Cys113 and Cys190 localized in extracellular loops I and II, respectively, forms an essential disulfide bond for high-affinity melatonin binding to MT₂ and possibly to MT₁ receptors as well [85]. Furthermore, using chemical modification with thiol-reducing and thiol-alkylating agents and site-directed mutagenesis, we reported that Cys140 regulates binding affinity while Cys143 and Cys219 determine binding capacity to the human melatonin MT₂ receptor [85]. In conclusion cysteines located in MT₂ receptor regions near the ligand binding site and/or G-protein coupling regions are involved in *N*-ethylmaleimide-induced changes in affinity and receptor density.

Site-directed mutagenesis revealed unique structural features within the MT₁ and MT₂ melatonin receptor molecular structure which show potentially distinct binding pockets for ligand recognition. Various models for melatonin recognition at melatonin receptors have been proposed based on the amino acid sequences of the melatonin receptor types (MT₁, MT₂, and Mel_{1c}), the helical structures of bovine rhodopsin [86] and bacteriorhodopsin [87, 88], and radioligand affinity constants (for a review see [89–92]. The conserved histidine in TM V is critical for binding to both the MT₁ and MT₂ melatonin receptors [90, 93, 94], which is in agreement with early models [86, 88]. Point mutations of conserved serines (S8 and S12) in TM III [95] and glycine G20 in TM VI [96, 97] are important for ligand binding to MT₁ receptors. In contrast, serines (S8 and S12) in TM III appear not to be involved in ligand binding to hMT₂ receptors, revealing potential structural differences between the binding pockets of the mammalian MT₁ and MT₂ melatonin receptors [90, 95]. Molecular modeling studies of the hMT₂ receptor revealed the involvement of Val204, Leu272, and Tyr298 within TM V, VI, VII, respectively as essential for ligand binding [92]. Thus, the binding pockets of the MT₁ and MT₂ melatonin receptors appear to share a common histidine residue (His7 in TM5) but also have distinct residues (Ser8 and Ser12 in TM3) necessary for ligand binding [89–91]. These differences in the molecular structure of the binding sites of the MT₁ and MT₂ melatonin receptors could be used in the design of selective and novel molecules with therapeutic potential.

2.4.2 Molecular Pharmacology

Identification and characterization of specific melatonin receptor responses in native tissues requires the use of well-characterized melatonin receptor ligands and radioligands in terms of pharmacological properties (affinity, selectivity, specificity) and ligand efficacy (agonist, competitive antagonist, partial agonist, inverse agonist) [19, 98]. This information is important as the efficacy of GPCR ligands to initiate a signaling response in target tissues is dependent on the cellular milieu, G-protein-type and/or scaffolding molecules, spare receptors, and dimer and/or heterodimer formation (for a review see [19, 99]. A ligand is considered selective on a specific receptor when its affinity and/or potency is at least 100 times higher with respect to the other receptor under consideration [53].

Prototype nonselective MT₁/MT₂ melatonin receptor ligands currently used to identify melatonin receptor-mediated responses include several agonists [e.g., 6-chloromelatonin, agomelatine (S20098)] and competitive receptor antagonists (e.g., luzindole, S20928) [52, 58, 60] (Fig. 2.2). Luzindole has 15- to 26-fold higher binding affinity for the MT₂ receptor, with an affinity constant (K_B) assessed by Schild

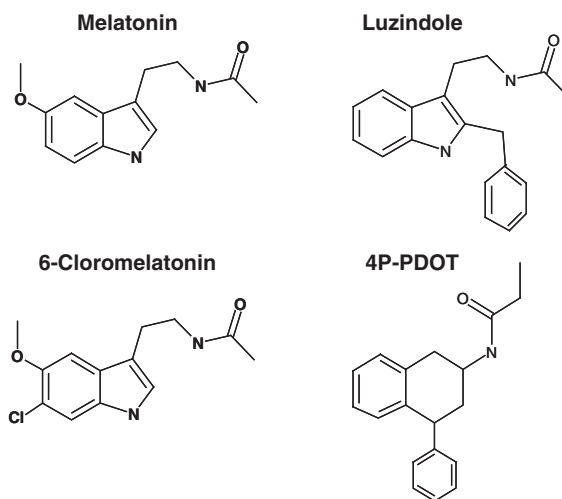


Figure 2.2 Chemical structures of melatonin and prototype melatonin receptor agonists and antagonists: melatonin, 5-methoxy-*N*-acetyltryptamine; 6-chloromelatonin; 6-chloro-5-methoxy-*N*-acetyltryptamine; luzindole, 2-benzyl-*N*-acetyltryptamine; 4P-PDOT, 4-phenyl-propionamide tetraline.

analysis in retina of 20 nM. The affinity constant (K_B) of luzindole to compete for melatonin-mediated vasoconstriction in caudal artery is 156 nM [52, 100]. 4P-PDOT (4-phenyl-2-propionamidotetraline) is a selective MT_2 ligand as it shows over 300 times higher binding affinity for the MT_2 than the MT_1 melatonin receptor with an affinity constant (K_B) for MT_2 melatonin receptors in retina 0.5 nM [52, 55]. Of interest is the observation that 4P-PDOT acts as a partial agonist on MT_2 melatonin receptors in the rat microcirculatory system [101] and in heterologous cells expressing recombinant receptors [90]. Luzindole and 4P-PDOT are also MT_1 melatonin receptor antagonists at 300 nM and higher concentration; however, they both act as inverse agonists in systems endowed with constitutive active MT_1 receptors [55, 102–104]. Other selective MT_2 melatonin receptor ligands characterized as competitive MT_2 receptor antagonists have been recently described [e.g. 4P-CADOT (4-phenyl-2-chloroacetamidomidotetraline), 4P-ADOT (4-phenyl-2-acetamidotetraline), K185 (*N*-butanoly-2-(5,6,7-trihydro-11-methoxybenzo[3,4]cyclohept [21a] indol-13-yl)ethanamine), GR128107 (3-(1-acetyl-3-methyl-piperidine) 5-methoxyindole, and 5-methoxyluzindole)] [52, 55, 105]. The ligand IIK7 is a selective MT_2 melatonin receptor agonist at recombinant receptors [105]. Recently, the synthesis and pharmacological characterization of S26131 [*N*-(2-{7-[3-({8-[2-acetylamin]ethyl}-2-naphthyl)oxy]propoxy}-1-naphthyl)ethyl)acetamide] which shows over 200-fold higher affinity for the MT_1 than the MT_2 melatonin receptor, was reported [57]. This molecule was synthesized by dimerization of two molecules of S20098 linked together by three methylene groups through the methoxy substituents [57]. S26131 antagonized melatonin-mediated stimulation of ^{35}S -GTP γ S [(35S)GTP γ S]; guanosine 5'-O-(3-[35S]thio)triphosphate] binding to MT_1 receptors expressed in heterologous mammalian cells with only 27 times higher affinity for the MT_1 than for the MT_2 receptor [57]. The efficacy of this putative MT_1 melatonin receptor antagonist in tissues expressing native melatonin receptors has not been reported.

GPCRs form homodimers and heterodimerize with other members of the same receptor superfamily. Oligomerization is a general phenomenon that regulates

trafficking, ligand binding, and signaling of several GPCRs, including opiate, muscarinic, α -adrenergic, and γ -aminobutyric acid (GABA) receptors [106–109]. Oligomerization and receptor internalization and/or desensitization are mechanisms by which melatonin regulates its receptors. Regulation of melatonin receptors by oligomerization is supported by several findings. First, isolation of solubilized ligand–melatonin receptor complexes by gel filtration chromatography yield molecular sizes of 110 or 150 kDa [110, 111], which corresponds to receptor trimers and tetramers, respectively, and show low affinity for 2- 125 I]iodomelatonin binding [110, 111]. Second, Ayoub et al. [112] reported constitutive dimerization and oligomerization of hMT₁ and hMT₂ melatonin receptors expressed in human embryonic kidney (HEK) 293 cells using both immunoblots and bioluminescent resonance energy transfer (BRET). Melatonin, neutral antagonists, and inverse agonists all promoted increases in BRET values for the MT₂ but not the MT₁. More recently, Ayoub et al. [113] demonstrated, using cell lines expressing low levels of MT₁ and/or MT₂ melatonin receptors and BRET to assess the pharmacological ligand binding profile, that the probability of MT₁/MT₂ heterodimer formation is similar to or even higher than those of the corresponding homodimers. Interestingly, both the MT₁ and MT₂ binding sites are functional within the heterodimer and the corresponding binding sites maintain their respective selectivity for MT₁ and MT₂ selective ligands. Together, these results suggest that oligomerization and heterodimerization of melatonin receptors may modulate trafficking and signaling responses by melatonin, which is an important factor to be taken into account when the pharmacology of new ligands is assessed.

2.4.3 Signaling

Mammalian MT₁ melatonin receptors signal through activation of pertussis toxin–sensitive and pertussis toxin–insensitive cellular pathways [47, 114]. Activation of G_i-coupled (G α_{i2} and G α_{i3}) MT₁ melatonin receptors inhibits forskolin-stimulated cAMP formation, protein kinase A (PKA) activity, and phosphorylation of the cAMP-responsive element binding protein (CREB) [115–117] (Fig. 2.3a). In recombinant systems, activation of the MT₁ melatonin receptor, via the release of the $\beta\gamma$ subunit, activates a Kir3 potassium channel and potentiates PGF_{2 α} stimulation of phospholipase C (PLC) activation and arachidonic acid release through a PTX (Pertussis toxin)-sensitive G protein [116, 118, 119]. MT₁ melatonin receptors also couple to stimulation of PLC-dependent signaling cascades either directly through G_q-mediated increases in phosphatidylinositol turnover and/or indirectly via G $\beta\gamma$ -mediated increases in intracellular calcium [116, 120] (Fig. 2.3a). In MCF-7 (Human Breast Adenocarcinoma-7) breast carcinoma cells and heterologous systems expressing MT₁ receptors activation of MT₁ melatonin receptors stimulates c-Jun N-terminal kinase activity via both pertussis toxin–sensitive (G α_i) and pertussis toxin–insensitive G (G α_s , G α_z , and G₁₆) proteins [121] and increases phosphorylation of MEK (mitogenic activated protein kinase-MAPK/extracellular regulated kinase-ERK kinase) 1 and 2 and extracellular regulated kinase (ERK) 1 and 2 [121, 122].

The MT₂ melatonin receptor expressed in heterologous cells inhibits forskolin-stimulated cAMP accumulation and decreased cGMP formation via the soluble guanylyl cyclase pathway and increases phosphoinositide turnover [51, 123, 124] (Fig. 2.3b). Activation of MT₂ melatonin receptors stimulates PKC activity in the rat SCN [125] and inhibits GABA_A receptor-mediated currents in the hippocampus [126].

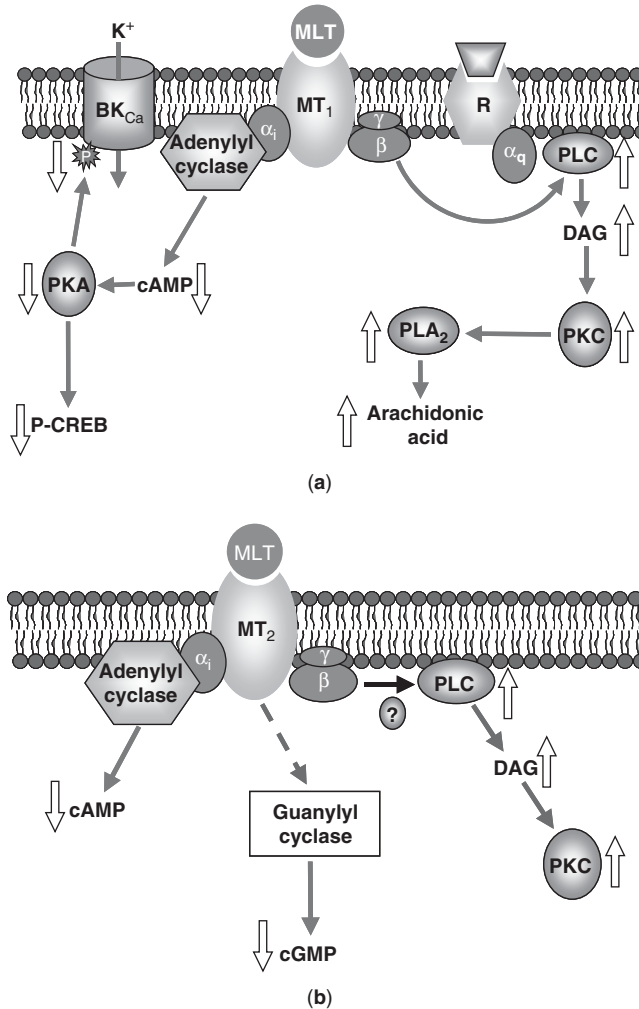


Figure 2.3 Signaling events mediated by activation of MT₁ and MT₂ melatonin receptors. (a) Melatonin (MLT) signals through activation of MT₁ melatonin receptor via two parallel pathways mediated by α-subunit (i.e., inhibition of cAMP formation) and βγ subunits [i.e., potentiation of phosphoinositide turnover stimulated by a G_q-coupled receptor (R)] of G_i. (b) Signaling pathways coupled to MT₂ melatonin receptor activation. Melatonin-mediated phase shifts of circadian rhythms through MT₂ receptors are mediated by protein kinase C (PKC) activation (the mechanism leading to PKC activation remains putative, however). DAG, diacylglycerol; BK_{Ca}, calcium-activated potassium channel; cGMP, cyclic guanosine monophosphate; R, G_q-coupled receptor (i.e., prostaglandin PGF_{2α} receptor FP and purinergic receptor P2Y). (Reprinted from [71] with permission from Elsevier.)

In the rabbit retina the presynaptic melatonin heteroreceptor inhibiting dopamine release possesses the pharmacological characteristics of the MT₂ receptor [13, 52].

Melatonin receptor-mediated vasoconstriction and vasodilation through activation of MT₁ and MT₂ melatonin receptors, respectively, have been reported in peripheral and in cerebellar arteries of various species, including human [100,

127]. In smooth muscle, activation of MT₁ melatonin receptors potentiates α -adrenergic-mediated vasoconstriction by blocking BK_{Ca} via activation of G_i/G_o-protein-coupled MT₁ melatonin receptors and decreases in cAMP-mediated phosphorylation [128] (Fig. 2.3a). Participation of receptors localized in the endothelium in this response cannot be ruled out [100, 128, 129]. Melatonin-mediated vasoconstriction of cerebral arteries is blocked by the competitive melatonin receptor antagonists luzindole and S20928, by pertussis toxin, and by blockers of BK_{Ca} channels [130–132]. MT₁ melatonin receptor activation may regulate cerebral blood flow [127] as this receptor has been localized by immunohistochemistry in the arterial wall and hippocampal microvasculature of normal and Alzheimer's disease subjects. Vasoconstriction induced by melatonin may modulate vascular tone and attenuate diurnal fluctuations in blood pressure, keeping cerebral flow constant [133]. Increases in MT₁ receptor immunoreactivity in cerebral vessels from Alzheimer's subjects may result from decreases in melatonin levels associated with age and cerebral hypofunction associated with neurodegeneration [127].

Melatonin receptor-mediated vasodilation was demonstrated in rat arteries. Potentiation of phenylephrine-induced contractions by melatonin in caudal arteries is enhanced in the presence of the MT₂ selective receptor antagonists 4P-PDOT, suggesting blockade of a receptor involved in decreasing vascular tone, possibly the MT₂ receptor [100, 134]. Vasodilation and increase in blood flow induced by melatonin in distal skin regions may underlie the concomitant heat loss and hypothermic effect of this hormone [135]. Estrogens modulate melatonin receptor-mediated changes in vascular contractility. The vascular reactivity to melatonin varies during the estrous cycle, being reduced during proestrous, when the levels of estrogens are high as compared with other stages of the estrous cycle and following ovariectomy [136]. MT₁ and MT₂ melatonin receptors are also differentially regulated at the various stages of the estrous cycle and following treatment with 17 β -estradiol [103, 104]. 17 β -Estradiol increases the vasodilatory effect of melatonin through changes in MT₁- and/or MT₂-mediated sensitivity [104, 136]. Furthermore, estrogen appears to regulate the state of activation as recombinant MT₁ receptors expressed in heterologous cells and treated with 17 β -estradiol, and native ovarian MT₁ receptors during proestrous [104] are found to be constitutively active [103, 104]. Together, these reports suggest that either endogenous or exogenous estrogens may differentially regulate melatonin receptor function in mammals.

2.5 THE SUPRACHIASMATIC NUCLEUS

2.5.1 Circadian Inputs and Outputs

The mammalian circadian timing system formed by the retina, the intergeniculate leaflet (IGL), and the SCN facilitates adaptation of the organism to environmental changes through the rhythmic regulation of behavior and physiological processes. The SCN, a pair of small clusters of cells located within the anterior ventral hypothalamic area just above the optic chiasm, times the near 24-h oscillations of behavior, physiology, metabolic, and neuroendocrine function [137–139]. The SCN receives projections from the retina, directly through the retinohypothalamic tract and indirectly through the IGL and from numerous other brain regions, integrating

incoming signals and generating overt circadian rhythms [140, 141]. The SCN controls the circadian rhythm of melatonin synthesis by the pineal gland in both the presence and absence of light cues. In turn, pineal melatonin modulates clock function in the SCN through a direct action on two GPCRs, MT₁ and MT₂ [19].

Synchronization of endogenous circadian clocks to the 24-h period of the sleep–waking cycle occurs by the combined actions of neuronal transmission [142]: internal (e.g., GABA) and external (e.g., light) stimuli [138], secretion of SCN-regulated hormones (e.g., melatonin, corticosteroid) [143], and paracrine signals by secretion of tumor growth factor- α (TGF α) [144] and/or prokineticin (PK2) [145] that regulate motor activity and vasopressin (AVP) and vasoactive intestinal peptide (VIP) that regulate SCN output targets [142, 146].

2.5.2 Melatonin Receptors in SCN: Localization, Signaling, and Function

Biochemical, molecular, and functional evidence consistently suggests the presence of two melatonin receptors, MT₁ and MT₂, in the mammalian SCN. Expression of both the MT₁ and MT₂ melatonin receptor mRNAs in the rat and mouse SCN was demonstrated by reverse transcriptase polymer chain reaction (RT-PCR) and/or by in situ hybridization histochemistry using highly selective and specific digoxigenin-labeled oligonucleotide probes [50, 55, 61, 105, 125, 147–149]. Hybridization of mRNA with both the MT₁ and MT₂ antisense probes was observed within the cytoplasmic region of cells with neuronal morphology in rat SCN slices and SCN2.2 cells [125, 149] (Fig. 2.4). Expression of melatonin receptor proteins in the rodent and human SCN has been demonstrated by receptor autoradiography with 2-[¹²⁵I]iodomelatonin, Western blot analysis, and/or immunohistochemistry [150]; see also [52, 125, 149, 151, 152]. Western blot analysis with receptor-specific antiserum revealed expression of immunoreactive proteins of molecular size 37 kDa for both MT₁ and MT₂ melatonin receptors in rat SCN2.2 and SCN membranes [149]. These molecular weights correspond to those previously reported for the receptors in human kidney [153], rat hypothalamus and retina [154], and immortalized hypothalamic GT1–7 (gonatropin-releasing hormone neurons) cells [155]. In rat SCN membranes proteins of 55 kDa (MT₁) and 48 kDa (MT₂) may represent glycosylated forms of the receptors [149, 155]. Together these data demonstrated the expression of both MT₁ and MT₂ melatonin receptors in the mammalian SCN.

The MT₁ and MT₂ melatonin receptors expressed in the mammalian SCN are involved in acute inhibition of neuronal firing and phase shift of circadian rhythms. The mammalian SCN *in vitro* expresses a spontaneous circadian rhythm of neuronal firing rate with a peak of activity at CT 6 (circadian time 6; CT 12 onset of activity of host animal) or middle of the subjective day that persists *in vitro* for several cycles [139, 156, 157] (Fig. 2.5). Acute treatment with melatonin inhibits neuronal firing in the SCN, shifting the activity of the nucleus toward night levels [61, 158]. Rat SCN neurons show the highest sensitivity to melatonin's acute inhibition of neuronal firing between CT 12 and CT 15 [159], suggesting that endogenously produced melatonin may alter the state of excitability of neurons as the clock moves into the night. Melatonin inhibits neuronal firing in the rodent SCN through activation of MT₁ melatonin receptors as this effect was absent in the SCN of MT₁ knockout mice [61] and was not affected in the MT₂ knockout mice [62]. In the mouse SCN, melatonin inhibits pituitary adenylate cyclase-activating polypeptide (PACAP)-mediated

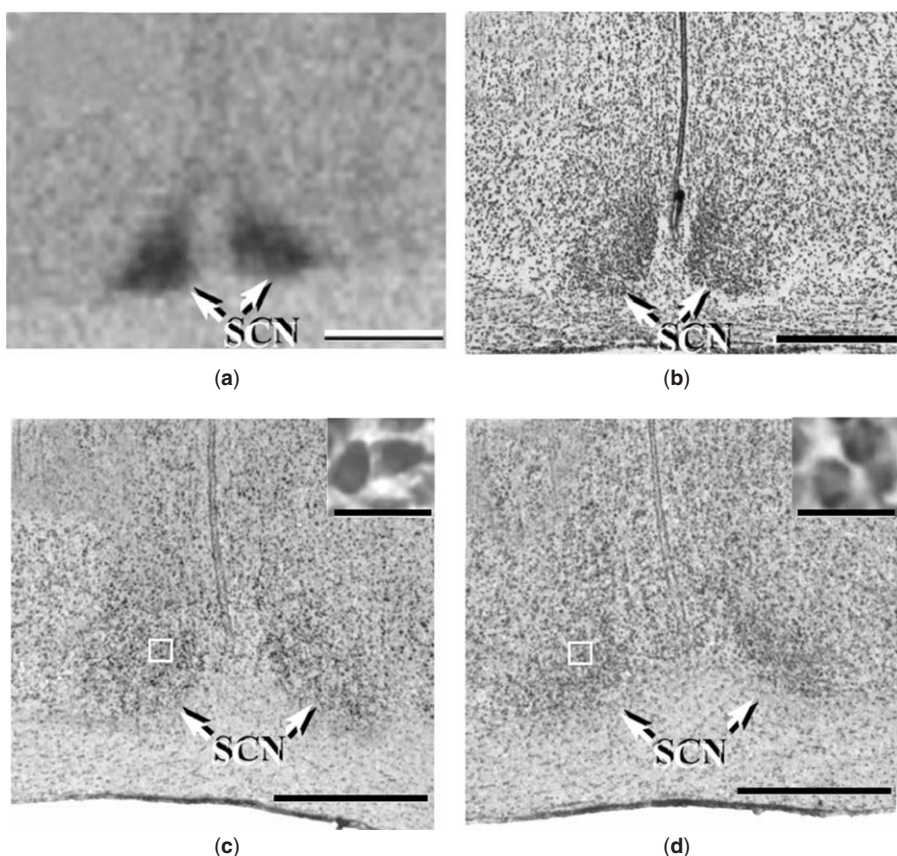


Figure 2.4 Melatonin receptor autoradiography and MT₁ and MT₂ mRNA expression in rat SCN. (a) Representative autoradiogram of 2-[¹²⁵I]iodomelatonin (2-[¹²⁵I]-IMLT) binding to rat SCN. To evaluate total binding (a), frozen coronal sections encompassing the SCN were incubated with 2-[¹²⁵I]iodomelatonin (200 pM). Nonspecific binding was determined by coincubation with melatonin (1 μM) and revealed no binding (data not shown). (b) Nissl stain of coronal section comprising SCN at similar magnification as (a). (c,d) Micrographs of hybridization signal for digoxigenin (DIG)-labeled MT₁ and MT₂ antisense oligonucleotide probes to rat SCN frozen sections. The hybridization signal was discretely localized throughout the SCN with both MT₁ antisense probe (c) and MT₂ antisense probe (d). No hybridization was detected with MT₁ and MT₂ sense probes (not shown). Scale bar, 0.5 mm. Insets: SCN cells taken from regions delineated by white squares in (c) and (d) at high magnification, demonstrating that hybridization to mRNA was restricted to the cytoplasm. Scale bar, 20 μm. [Modified (Figs. 1 and 2) from [125] with permission from the *American Journal of Physiology*.]

CREB phosphorylation [62, 160] through activation of MT₁ melatonin receptors, as these effects are not observed in the SCN from MT₁ knockout mice.

The master biological clock in the mammalian SCN is reset in response to melatonin at temporally distinct sensitive times, dusk (CT 9–11) and dawn (CT 22.2) (Fig. 2.5a) [138]. Melatonin and the MT₁/MT₂ nonselective melatonin analogs GR196429 (*N*-[2-[2,3,7,8-tetrahydro-1*H*-furo (2,3-*g*)indol-1-yl]ethyl]acetamide) [161] and S20098 (agomelatine) [162] induce a concentration-dependent phase advance of the circadian rhythm of neuronal firing when applied at CT 10 to the

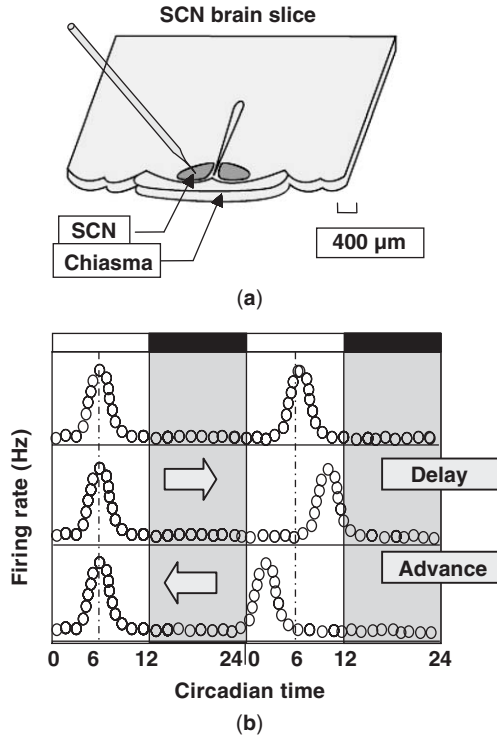


Figure 2.5 Circadian rhythms of neuronal firing in SCN brain slice. Schematic representation of a coronal slice (400 μm) of rodent brain containing SCN. The spontaneous rhythms of neuronal firing recorded from rodent SCN brain slices by extracellular recording follow a circadian pattern with a peak observed at mid-subjective day (CT 6–7) indicated by the dashed line. This peak can be used as a marker to assess the effect of various stimuli on clock phase. A *phase advance* of the peak is observed when the peak of neuronal activity appears earlier than in controls. A *phase delay* results from a stimulus that causes the peak of neuronal activity to occur later than in controls. The periods of sensitivity to induce phase advances or phase delays are unique for each stimuli and are constant on all mammalian species. (See color insert.)

rat and/or mouse SCN brain slice [125, 156, 163–165] (Fig. 2.5b). These melatonin-mediated phase advances are blocked by pertussis toxin and specific inhibitors of PKC [61, 156]. In the rat SCN brain slice activation of PKC by phorbol esters phase advance the circadian rhythm of neuronal firing when applied at either CT 10 or CT 23 [156]. Melatonin-mediated stimulation of PKC phosphotransferase activity at CT 10 is blocked by luzindole and MT_2 receptor selective concentrations of the antagonist 4P-PDOT [125]. Together, these data demonstrated that melatonin-mediated phase shifts of the circadian rhythm of neuronal firing are induced through activation of a G-protein-coupled PKC-mediated signaling pathway. The melatonin-mediated phase advances in rodent SCN brain slices are blocked by the MT_1/MT_2 competitive melatonin receptor antagonist luzindole [125, 164]. Melatonin application at CT 10 phase advances the circadian rhythm of neuronal firing in a concentration-dependent manner with identical potency in the SCN brain slice from both the wild-type and MT_1 knockout mice [61, 165]. These phase advances are completely blocked by MT_2 selective concentrations of the competitive

receptor antagonist 4P-PDOT [166]. Melatonin did not phase shift the circadian rhythm of neuronal firing in SCN brain slices from mice with genetic deletion of MT₂ receptors [167]. Taken together these results strongly suggest that activation of MT₂ melatonin receptors in the rodent SCN phase shift circadian rhythms of neuronal firing through an MT₂ receptor-mediated, PKC-dependent signaling pathway.

Immortalized cells from the rat SCN (SCN 2.2) retain most biochemical and biophysical characteristics of the native tissue, including the expression of clock genes, regulatory proteins, and rhythmic biological clock function [168, 169]. The SCN 1.4 and SCN 2.2 cell lines consist of a heterogeneous population of neuronal- and glial-like cells that show circadian patterns of 2-deoxyglucose uptake and neurotrophic factor 3 (NT-3) content, restored circadian wheel-running activity when transplanted into SCN-lesioned arrhythmic rats, and induced metabolic and molecular rhythmicity to cocultured NIH-3T3 fibroblasts and gated sensitivity to the glutamate- and melatonin-mediated phase shift of circadian rhythms [168–173]. Moreover, SCN 2.2 cells exhibit basal endogenous oscillations in PKC activity and modulation of PKC activity by melatonin at the same periods of sensitivity (CT 2 and CT 10) observed in the native SCN [125, 171]. SCN 2.2 cells expressed functional MT₁ and MT₂ receptors that localized onto cells with neuronal-like morphology [149]. Melatonin inhibits forskolin-stimulated cAMP formation through activation of G-protein-coupled melatonin receptors and stimulated PKC activity through activation of the MT₂ receptor [149]. Physiological concentrations of melatonin (300 pM) mimicking the nocturnal secretion (8 h) decreased MT₂ but not MT₁ melatonin receptor density in SCN 2.2 cells transiently expressing these receptors and desensitized endogenous MT₂ melatonin receptors coupled to PKC stimulation [173]. Together these results suggest that the melatonin-mediated inhibition of neuronal firing and of PACAP-induced CREB phosphorylation is mediated through activation of the MT₁ receptor while phase shifts of neuronal rhythms of firing rate in the SCN are mediated through activation of the MT₂ receptor. It is possible that the MT₁-mediated signaling pathway is involved in the regulation of circadian rhythms in output targets.

2.5.3 Melatonin Receptors and Clock Genes

Daily rhythms in behavior and physiology are driven by biological oscillators that synchronize endogenous rhythms with natural environmental cycles [137, 139]. The circadian signal is generated by autoregulatory transcriptional and translational feedback loops in the master biological clock as well as in other peripheral clocks in neuronal and nonneuronal cells. The CLOCK–BMAL1 transcription factors form heterodimers that drive the transcription of the mammalian period genes (*Per1*, *Per2*, *Per3*) and two cryptochrome genes (*Cry1*, *Cry2*) during the subjective day. In turn the Per and Cry proteins act as negative regulators of the feedback loop. The role of mPers is primarily to form complexes with mCRY and transport mCRY proteins into the nucleus [137, 174, 175]. Recently, a second, stabilizing feedback loop was characterized in mammals, where the nuclear receptors Rev-erb and ROR α and are respectively repressors and activators on the same RORE elements of the *Bmal1* promoter, generating the rhythms of *Bmal1* transcription [176, 177] (Fig. 2.1). The *timeless* (*Tim*) gene has an essential role in circadian clock function in *Drosophila*, while the putative mouse homolog *mTimeless* (*mTim*) is essential for embryonic

development [178]. The circadian function of mTIM in the mammalian clock has been controversial, as several publications reported no oscillation of *mTim* mRNA in the mammalian SCN [179, 180]. However, recently Tischkau et al. [181] reported a significant diurnal oscillation of *mTim* mRNA in the SCN. *mTim* appears to be essential for rhythmicity in the mouse SCN and it is part of the negative-feedback arm of the mammalian clockwork [182]. The short form of TIM (mTIM-s) is constitutively expressed in the SCN, while the full length (mTIM-fl) exhibits a diurnal oscillation [182]. Based on the time course of light-induced *mTim* and *mPer2* mRNA expression, it was suggested that mTIM interacts with mPER-s, with mPER2 being the preferred partner of mammalian TIM [182].

Photic and nonphotic stimuli modulates clock gene expression (e.g., *mPer1*, *mPer2*, and *mTim*) [76, 180, 181, 183–185]. Melatonin effects on clock gene expression and the molecular mechanism(s) by which the hormone changes clock phase have remained elusive. Most studies reported no change or very small changes on clock gene expression by melatonin in the rat SCN [186, 187]. Poirel and colleagues [187] observed changes in clock gene expression following melatonin administration to rats only on the second day after treatment. The fact that melatonin administration was outside the period of sensitivity for phase shift and conducted under a light–dark cycle together with a lengthy sampling interval may have contributed to the negative results. More recently, however, Isobe and Nishino (2004) [188] demonstrated increases in *mPer2* gene expression in the rat SCN 3 h after melatonin administration. Evidence suggests that *mTim* may be the clock gene regulated by melatonin. Disruption of SCN neuronal activity rhythms by application of antisense for the *mTim* clock gene phase shifts the clock generating a phase response curve of neuronal firing [182] that is opposite to the mammalian melatonin phase response curve [189], with maximum advances at CT 2–4 and phase at CT 10–12. Kandalepas et al. (2004) [190] reported that melatonin applied at CT 10 but not at CT 6 stimulated *mTim-fl* mRNA expression, an effect blocked by a PKC inhibitor. Together these results suggest a possible role for *mTim* in melatonin-mediated phase shift of circadian rhythms.

2.5.4 Regulation

Melatonin receptors, as other members of the GPCR superfamily, maintain timely cellular function and homeostasis by regulating signal transduction events through the process of desensitization and/or supersensitization. Desensitization is the waning of receptor responsiveness following persistent agonist challenge and can be characterized by uncoupling of receptor and G protein, receptor internalization, and/or receptor downregulation [109]. Supersensitization, by contrast, relates to enhanced responses generally resulting from persistent inhibition or blockade of a receptor [191].

In the rodent SCN, the density of endogenous melatonin receptors and mRNA expression follow either diurnal or circadian rhythms, with shapes varying among laboratories and experimental conditions, including sex, age, species, tissue, environmental conditions, and/or clock regulation [148, 192–194]. Specific 2-[¹²⁵I]iodomelatonin binding to the rodent SCN is attributed to MT₁ melatonin receptors, as the radioligand does not detect MT₂ binding sites and melatonin was shown to both positively and negatively regulate its own receptors. While radioligand binding to the rat SCN demonstrated an inverse relationship between receptor

density and serum melatonin levels [192, 195], other studies show higher receptor density at times when endogenous melatonin is high [47, 193]. Melatonin receptors expressed in the SCN appear to be regulated by endogenous melatonin, the master biological clock, and photic input from the retina [192–194, 196–198]. Interestingly, prolonged exposure to a concentration of melatonin mimicking nocturnal levels (400 pM) did not affect the number of recombinant MT₁ melatonin receptors expressed in mammalian cells, the affinity for melatonin, or the functional sensitivity of the receptor [90]. Together, these data suggest that the changes in MT₁ melatonin receptor sensitivity in the mammalian SCN may result from a combined effect of receptor activation by endogenous melatonin, crosstalk with receptors activated by photic input, and the circadian clock.

2.5.4.1 Desensitization. The MT₁ and MT₂ melatonin receptors are differentially regulated depending on the melatonin concentration used (physiological vs. supra-physiological), time of exposure, and cellular background. Prolonged exposure (8 h) of recombinant MT₁ receptors to supraphysiological concentrations of melatonin (100 nM) significantly increased the number of MT₁ melatonin receptors, decreased the affinity (K_i), and desensitized the MT₁ melatonin receptor-mediated stimulation of [³⁵S]GTPγS binding, without internalization of the receptor [199]. In contrast, prolonged exposure to a concentration of melatonin mimicking nocturnal levels (400 pM) did not affect the number of MT₁ melatonin receptors, the affinity for melatonin, or the functional sensitivity of the receptor [199]. Endogenous melatonin should not significantly affect the functional sensitivity of MT₁ melatonin receptors [199]; however, exogenous melatonin taken therapeutically at doses above physiological levels could desensitize the receptor, thereby affecting physiological responses mediated following activation of MT₁ melatonin receptors [200].

The MT₂ melatonin receptor exhibits rapid desensitization and internalization upon exposure to both physiological and supraphysiological concentrations of melatonin [90, 91]. Pretreatment with physiological concentrations of melatonin (30–300 pM or 7–70 pg/mL) decreased the number of hMT₂ melatonin receptors heterologously expressed in mammalian cells in a time- and concentration-dependent manner [91]. Furthermore, hMT₂-GFP (human MT₂-Green Fluorescent Protein) melatonin receptors heterologously expressed in immortalized SCN 2.2 internalized upon pretreatment with both physiological (300 pM or 70 pg/mL) and supraphysiological (10 nM or 2.3 ng/mL) concentrations of melatonin. The decrease in MT₂ melatonin receptor number induced by melatonin (300 pM for 1 h) was reversible and reached almost full recovery after 8 h; however, after treatment with 10 nM melatonin full recovery was not attained even after 24 h. This recovery process was partially protein synthesis dependent [91].

The functional sensitivity of endogenous MT₂ melatonin receptors in the rat SCN was studied by determining the ability of melatonin applied at CT 23 to phase advance the circadian rhythm of neuronal firing following pretreatment with physiological concentrations of melatonin (30–300 pM or 7–70 pg/mL) for a length of time mimicking the nocturnal surge (8 h). This treatment desensitized the MT₂ receptors precluding phase advances of the peak of neuronal firing, as the peak position was not different from that observed in SCN slices treated with vehicle for 8 h (Fig. 2.6) [91]. This functional desensitization is mediated through activation of MT₂ receptors by melatonin as it was completely blocked by cotreatment with

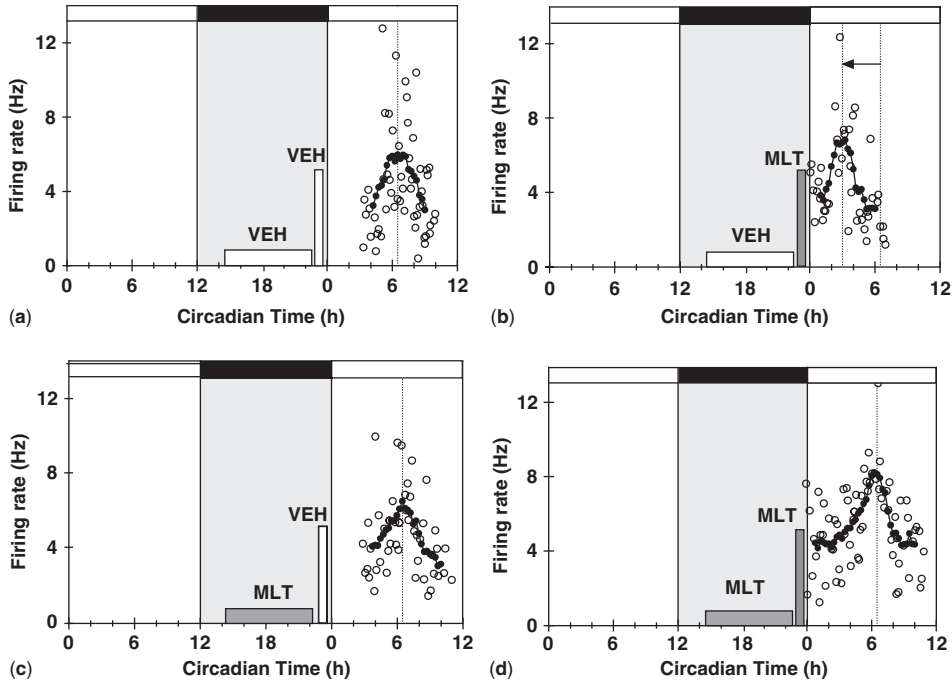


Figure 2.6 Desensitization of endogenous MT₂ melatonin receptors in rat SCN impaired melatonin-mediated phase shifts. Shown are representative rhythms of neuronal firing assessed by extracellular recording in rat coronal brain slices containing the SCN. The horizontal bar indicates pretreatment during subjective night with vehicle (CT 14.5–22.5) and melatonin (300 pM, 8 h) (CT 14.5–22.5). The vertical bar represents stimulation with either vehicle or melatonin (1 nM) applied by a 1-μL microdrop for 10 min at CT 23. Firing rates from individual cells in the SCN (open circles) are shown as well as the mean firing rates presented as running averages (filled circles). Phase shifts were visually determined with reference to the peak of the circadian rhythm of neuronal firing at CT 6.8 ± 0.1 ($n = 3$) (a) obtained after pretreatment with vehicle for 8 h and a vehicle pulse at CT 23. VEH: vehicle; MLT: melatonin. [Modified (Fig. 8) from [173] with permission from the *FASEB*.]

the MT₂ melatonin receptor antagonist 4P-PDOT [91]. Similarly, in rat immortalized SCN 2.2 cells, basal PKC activity shows a diurnal rhythm with nadirs during subjective day at CT 10 and CT 2 [171]. At these periods of sensitivity, melatonin stimulates PKC activity through activation of MT₂ melatonin receptors. Physiological concentrations of melatonin mimicking the nocturnal duration in vivo, desensitized the MT₂ melatonin receptor-mediated activation of PKC at CT 2. This effect was blocked by the competitive MT₂ melatonin receptor antagonist 4P-PDOT [91]. In vivo the nightly secretion of melatonin desensitizes endogenous MT₂ melatonin receptors in the mammalian SCN, thereby providing a temporally integrated profile of sensitivity of the mammalian biological clock to a melatonin signal.

2.5.4.2 Supersensitization. Activation of hMT₁ melatonin receptors by physiological concentrations of melatonin for a length of time mimicking the nocturnal surge increased hMT₁ melatonin receptor binding and induced functional supersensitiza-

tion of the cAMP signaling system upon withdrawal of the hormone [117, 201, 202]. This includes increase in cAMP formation, activation of protein kinase A, and phosphorylation of the transcription factor CREB [117, 203]. Indeed the circadian release of melatonin during the hours of darkness, through activation of high-affinity MT₁ melatonin receptors in target tissues, may inhibit stimulated cAMP formation resulting in supersensitization of cAMP signaling pathways linked to receptors coupled to G_s during the period of withdrawal [117, 201, 204]. It is therefore conceivable that melatonin-mediated supersensitization of the cAMP signaling pathway is a general mechanism by which the hormone transmits circadian signals through activation of CRE containing genes regulated by the transcription factor CREB [e.g., brain-derived neurotrophic factor (BDNF), *Per1* and *Per2* genes].

Clock genes are also found in many peripheral tissues, where the rhythms are delayed with respect to those observed in the SCN [137]. Peripheral rhythms of clock gene expression depend on neuronal and/or neuroendocrine output from the SCN [204, 205]. In the pars tuberalis (PT) and striatum, rhythms of *Per1* gene expression are dependent on the circadian melatonin signal possibly mediated through activation of MT₁ receptors in the target tissues [204, 206]. The PT of the pituitary rhythmically expresses *mPer1* and *mCry1* clock genes. In this tissue, melatonin increases *mCry1* expression during early subjective night while decreasing *Per1* expression during early subjective day [207]. In the PT, melatonin appears to be involved in setting the gain for incoming stimuli into target tissues as it facilitates the effect of adenosine on *mPer1* expression through supersensitization of adenylyl cyclase effect that is absent in the SCN [186]. A similar mechanism of adenylyl cyclase supersensitization leading to increasing cAMP production and insulin secretion has been described in pancreatic β cells upon prolonged melatonin pretreatment [208]. The circadian signal generated within the SCN and transmitted via the release of pineal melatonin may be the mechanism by which the master clock sends light and dark signals to other brain or peripheral tissues.

2.6 MELATONIN MODULATION OF CIRCADIAN RHYTHMS OF BEHAVIOR

Melatonin-mediated phase shifts of circadian rhythms occur at two windows of sensitivity that correspond to the hours around the day–night (dusk) and night–day (dawn) transitions [18, 55, 189, 209, 210]. Melatonin administered at the end of subjective day in vivo phase advances the onset of melatonin production in humans [18, 211–213] and of circadian activity rhythms in rodents [165, 189]. Figure 2.7 shows the melatonin-mediated phase advance of activity rhythm onset in both “melatonin-deficient” (C57BL/6) and “melatonin proficient” (C3H/HeN) mice [165, 214, 215]. Furthermore, in both mice and humans melatonin administered at the night–day transition phase delays circadian activity rhythms [189, 209]. As demonstrated with melatonin the nonselective MT₁/MT₂ melatonin receptor analogs S20098 and TAK 375 (Fig. 2.8) phase shift circadian rhythms of activity and accelerate reentrainment after a phase advance of the dark cycle in rodents [165, 216–219].

Pharmacological studies suggest that the melatonin-mediated phase shifts of circadian activity rhythm onset in the mouse in vivo [55] and of neuronal firing in the rat SCN brain slice in vitro [61, 91, 125, 156, 165] are mediated through activation

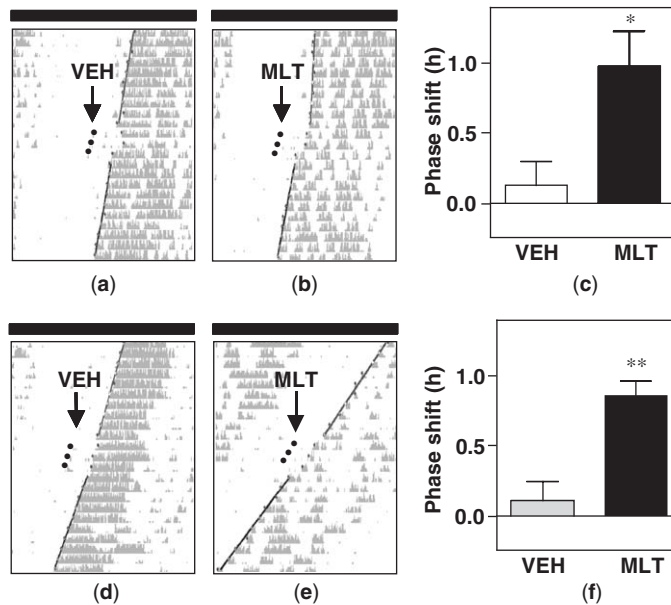


Figure 2.7 Melatonin phase advanced onset of wheel-running activity rhythms in C3H/HeN and C57BL/6 mice. Representative wheel-running activity actograms of individual C3H/HeN (a, b) or C57BL/6 (d, e) mice treated with vehicle (a, d) or melatonin (b, e). Wheel-running activity rhythms were recorded from mice kept in constant dark conditions. Mice were treated with either vehicle (3% ethanol/saline, s.c.) [(c) $n = 8$; (f) $n = 8$] or melatonin (90 $\mu\text{g}/\text{mouse}$, s.c.) [(c) $n = 10$; (f) $n = 8$] at CT 10 for three consecutive days. Black dots point to treatment time each day. (c, f) Bars represent mean \pm standard error of the mean (SEM) of phase shift of activity onset expressed in hours for vehicle- (VEH) and melatonin (MLT) treated C3H/HeN (c) and C57BL/6 (f) mice. (*) $P < 0.05$; (**) $P < 0.01$ when compared with vehicle treated. [Modified (Figs. 1 and 3) from [165] with permission from Blackwell Publishing.] (See color insert.)

of melatonin receptors. MT_2 selective concentrations of the competitive antagonist 4P-PDOT blocked melatonin-mediated phase shift of the circadian rhythm of neuronal firing in rat and mice SCN brain slices [125, 165, 166]. Furthermore, melatonin advances the peak of circadian rhythm of neuronal firing with identical potency in the SCN brain slices from either wild-type or MT_1 knockout mice, strongly suggesting the involvement of the MT_2 melatonin receptor [165]. Paradoxically, however, melatonin did not phase shift circadian rhythms of activity or accelerated reentrainment to a new dark onset after an abrupt advance of the dark cycle in C57B6/J mice [165]. Together, these data suggest that in vivo activation of molecular events regulated through MT_1 melatonin receptors within or outside the SCN and/or in output pathways may be necessary and perhaps sufficient for the expression of melatonin-mediated phase shifts of circadian rhythm of locomotor activity.

The pineal gland, through the release of the hormone melatonin, contributes to the integrity of circadian rhythmicity in mammals. This hypothesis is supported by reports demonstrating that daily melatonin administration enhances the organization of disrupted circadian rhythms [220], pinealectomy facilitates the loss of circadian rhythmicity in rodents kept in constant light [221], and melatonin administration in

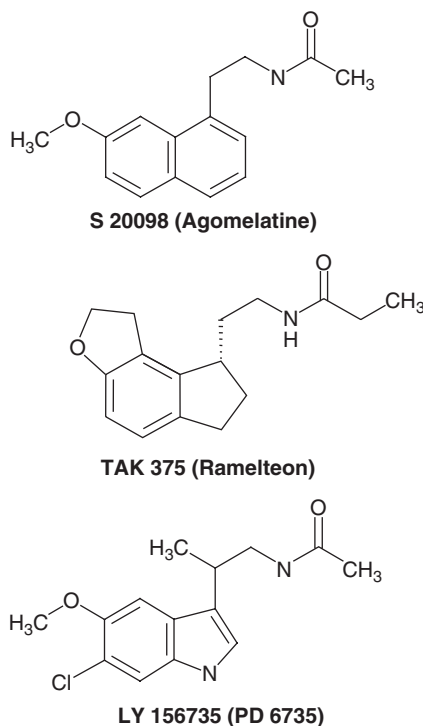


Figure 2.8 Melatonin receptor ligands under investigation for treatment of insomnia, circadian sleep disorders, and depression: **S20098 (agomelatine, Valdoxan, Institut de Recherches Internationales Servier)**, *N*-[2-(7-methoxy-1-naphthyl)ethyl] acetamide; **TAK 375 (ramelteon, Rozerem, Takeda Pharmaceutical)**, (*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno-[5,4-*b*]furan-8-yl)ethyl] propionamide; **LY 156735 (PD 6735, Eli Lilly and Co under development by Phase 2 Discovery)**, (*R*)-*N*-[2-(6-chloro-5-methoxy-1*H*-indol-3-yl) propyl]acetamide.

the diet corrects age-related changes in circadian response to environmental stimulus [222]. Additionally, nighttime melatonin appears to amplify and even restore disturbed circadian rhythms. In rats, daily melatonin administration amplifies the circadian rhythm of pineal norepinephrine content [223] and melatonin secretion [9] and restores the low-amplitude circadian rhythms of norepinephrine and acetylcholine synthesis in sympathetic ganglia, heart, and adrenal of aged rats [224] and the dampening of circadian rhythms of core body temperature induced by continuous illumination in rats [225]. The restored circadian rhythm of core body temperature persisted for several weeks after the last injection, suggesting that the synchronizing effect of melatonin resulted from its effect on SCN function. In humans, nighttime melatonin treatment amplifies the endogenous circadian melatonin secretion [226] and decreased blood pressure in male patients with essential hypertension [227]. Furthermore, melatonin supplementation increased nighttime rapid-eye-movement (REM) sleep and decreased core body temperature in patients with neuropsychiatric sleep disorders [228]. Together, these studies suggest that melatonin in addition to phase-shift circadian rhythms may play an important role in maintaining the integrity and amplitude of circadian rhythms of physiology.

2.7 MELATONIN RECEPTORS AS THERAPEUTIC TARGETS

Melatonin receptors are targets for the development of synthetic analogs to treat circadian and noncircadian sleep disorders. Currently three different nonselective MT₁/MT₂ melatonin receptor agonists (agomelatine, ramelteon, PD 6735) are under development for the treatment of insomnia, circadian rhythm disturbances, and depression (Fig. 2.8). The pharmacological properties as well as the potential therapeutic applications of these new investigational drugs are briefly described below.

2.7.1 Sleep

In humans the hormone melatonin produced in a circadian pattern with high levels at night promotes sleep at circadian phases when the SCN stimulates waking [17]. The observation that melatonin production is concurrent with nocturnal sleep and that the onset of evening melatonin production correlates with evening self-reported sleepiness and increased sleep propensity led to the hypothesis that melatonin and related ligands can be used to treat insomnia [17]. Insomnia is associated with daytime sleepiness and decrements in mental and physical performance. Involuntary insomnia occurs quite frequently due to endogenous or exogenous causes which do not allow sleep during the desired normal sleep time.

In normal volunteers physiological doses of oral melatonin (below 0.5 mg) which raise endogenous levels of the hormone to nocturnal levels are effective in decreasing sleep onset latency, oral temperature, and alertness [8, 35, 229]. Melatonin also promoted sleep in diurnal nonhuman primates and in human subjects suffering from age-related insomnia [200, 230, 231]. The sleep-promoting effects of melatonin are observed within an hour of administration, are independent of time of day, and are not mediated through interaction with benzodiazepine receptors [35, 232, 233]. Melatonin-mediated sleep promotion in humans is probably mediated through activation of melatonin receptors in the SCN and/or areas of the limbic system. Rodent studies suggest that melatonin-mediated decreases in neuronal activity are due to activation of MT₁ receptors [19, 61]. However, support for the hypothesis that this receptor is involved in melatonin-mediated sleep promotion in humans requires further investigation using pharmacological agents selective for the melatonin receptor types.

Ramelteon is a novel and nonselective high-affinity MT₁ and MT₂ melatonin receptor agonist with low affinity for the MT₃ binding sites that was developed for the treatment of insomnia and circadian sleep disorders [234] (Fig. 2.8). In cats [235] and monkeys [236] ramelteon promotes sleep, showing higher efficacy than melatonin. Ramelteon does not affect learning, memory, or motor function and is devoid of rewarding properties [237]. In humans with primary chronic insomnia, this novel melatonin receptor agonist decreases the latency to persistent sleep, increased total sleep time, and sleep efficiency [238]. The melatonin analog agomelatine, under development for the treatment of depressive disorders, also increases fatigue and reduces sleep latency in human volunteers [239]. In summary, melatonin as well as novel synthetic melatonin can induce physiological sleep without the common side effects frequently observed with sleep medication.

In subjects with moderate to severe sleep insomnia PD 6735 at 20, 50, and 100 mg in two consecutive days reduced sleep latency to persistent sleep as determined by polysomnography and reduced subjective sleep latency. PD 6735 did not negatively

affect other sleep parameters or produce morning-after psychomotor impairment [240]. PD 6735 does not show hypothermia, hypertension, and bradycardia, which are common side effects of melatonin [241].

2.7.2 Circadian Rhythms

Alterations between the timing of the endogenous sleep–wake cycle and the environmental light–dark cycle under conditions in which the sleep mechanisms are functionally intact lead to the so-called circadian sleep disorders. Circadian sleep disorders that involve alteration of the phase of the circadian clock with respect to the environment can be treated by timely administration of light or agents such as benzodiazepines or melatonin (for a review see [18]). These circadian sleep disorders include jet lag due to rapid crossing of time zones, shift work associated with changing work schedules, advanced sleep phase syndrome (ASPS) characterized by early sleep onset and offset of sleep, delayed sleep phase syndrome (DSPS) characterized by abnormal delay of sleep onset, and walking, non-24-h sleep–wake disorder characterized by irregular sleep–wake patterns [18]. Circadian sleep disorders also occur as a result of an abnormal light–dark cycle precluding the entrainment of endogenous rhythms to the 24-h period due to absence of environmental cues or a physical condition that altered the perception of light by the circadian system (e.g., blind individuals, tumor in the SCN). Treatment of circadian sleep disorders include chronotherapy [242], early-morning and early-evening light treatment [243, 244], and evening oral melatonin [245, 246].

In preclinical studies with normal volunteers agomelatine was found to phase shift circadian sleep patterns. Agomelatine administered in the early-evening phase advances core body temperature rhythms and increases the duration of REM sleep [239]. It also advances sleep termination, a phenomenon also observed upon administration of melatonin to subjects with DSPS [246]. Agomelatine advances the circadian phase of the salivary melatonin rhythm as well as the nocturnal regulation of core body temperature [239]. In these paradigms the effect of agomelatine was more pronounced with an oral dose of 100 mg, while melatonin was effective at a dose of 5 mg. PD 6735 was shown to accelerate readaptation of circadian rhythms following a stimulated 9-h phase advance in normal volunteers maintained in isolation [247]. Together, these studies demonstrate that these novel investigational new drugs have potential for the treatment of circadian rhythm disturbances.

2.7.3 Depression

Major depressive disorder is a common and serious psychiatric illness affecting between 10 and 15% of the adult population. The treatment of choice for this disorder is antidepressant medication. The introduction of selective serotonin reuptake inhibitors (SSRIs) has revolutionized treatment of depression providing efficacious therapy with lower toxicity and generally more tolerable side effects. Current investigational new treatments for depression use molecules with novel mechanism(s) of action, including pharmacological actions at melatonin receptors [99].

The melatonin system appears to be involved in the pathophysiology of affective disorders, namely depression. Melatonin and ligands with related pharmacological properties promote sleep and synchronize circadian rhythms, which are generally altered in major depression [248–250]. Dysregulation of the circadian release of

melatonin has been correlated with depressive states in clinical populations [251, 252]; however, it is unclear whether high or low melatonin levels, advanced or delayed phase, or amplitude irregularities contribute to depressive etiology or symptomology [253–258].

Melatonin and melatonin ligands exert antidepressant-like effects in several animal models of depression. Antidepressant treatment potentiates noradrenergic stimulation of pineal β adrenoceptors and hence melatonin production [258, 259]. Pharmacological doses of melatonin induce antidepressant-like effects in both mice and rats following a single or multiple swimming sessions [260–263] and in chronic mild stress paradigms in which sleep rhythms are disrupted [264]. The beneficial effects of melatonin in animal models of depression were strengthened by studies showing antidepressant-like effects of synthetic melatonin ligands. Overstreet et al. [265] reported that chronic but not acute administration of the melatonin receptor agonist S20304 (*N*-(2-(1-naphthyl)ethyl)cyclobutanecarboxamide) induced antidepressant-like effects in Flinders sensitive line (FSL) rats, which display innate high levels of immobility. Additionally, chronic administration of agomelatine, which activates both MT₁ and MT₂ melatonin receptors and blocks 5-HT_{2C} and 5-HT_{2B} receptors, induces antidepressant-like effects in rodents during forced swimming [263]. In contrast, melatonin shows no effect upon acute treatment or increased immobility in the swimming test [266, 267]. Interestingly, we demonstrated that the competitive melatonin receptor antagonist luzindole significantly decreased immobility in the swimming test [267]. The antidepressant-like effect of luzindole was observed in male and female C3H/HeN mice during the light and dark phases and was blocked in animals treated with melatonin [267, 268]. Luzindole decreases immobility during swimming in C3H/HeN wild-type mice and in mice with genetic deletion of MT₁ melatonin receptors but not in mice lacking the MT₂ receptor [268] (Fig. 2.9). These results suggest that luzindole may exert antidepressant-like effects through an action at MT₂ rather than MT₁ melatonin receptors.

Recently, attention focused on the antidepressant properties of agomelatine, a synthetic analog of the neurohormone melatonin that shows MT₁/MT₂ receptor agonist pharmacological properties and is a competitive 5-HT_{2C} and 5-HT_{2B} receptor antagonist [41, 269]. Studies in animals suggest that agomelatine may exert antidepressant and anxiolytic properties by a combination of action on MT₁ and MT₂ melatonin receptors and 5-HT_{2C} receptors [41, 270]; however, which is the relative contribution of each target to the overall effect is not known.

Agomelatine is a new antidepressant with a unique pharmacological profile that combines potential therapeutic actions on sleep, circadian rhythm dysfunction, and serotonin neurotransmission, which are known to be altered in depression. Agomelatine is significantly more effective than placebo in the treatment of major depression. At a dose of 25 mg agomelatine significantly reduced the depression scores as determined by the 17-item Hamilton Rating Scale for Depression (HAM-D) and several other criteria [269, 271]. Agomelatine significantly reduced the HAM-D scores after two weeks of treatment, showing higher efficacy than paroxetine, which was effective after four weeks treatment [271]. Both agomelatine and paroxetine were effective in the treatment of anxiety symptoms associated with depression [271]. Agomelatine is well tolerated with an adverse effect profile including discontinuation symptoms comparable to that of placebo and below that of paroxetine [271–273]. Paroxetine, an SSRI, has a half-life of 24 h while that of agomelatine is 2 h [272].

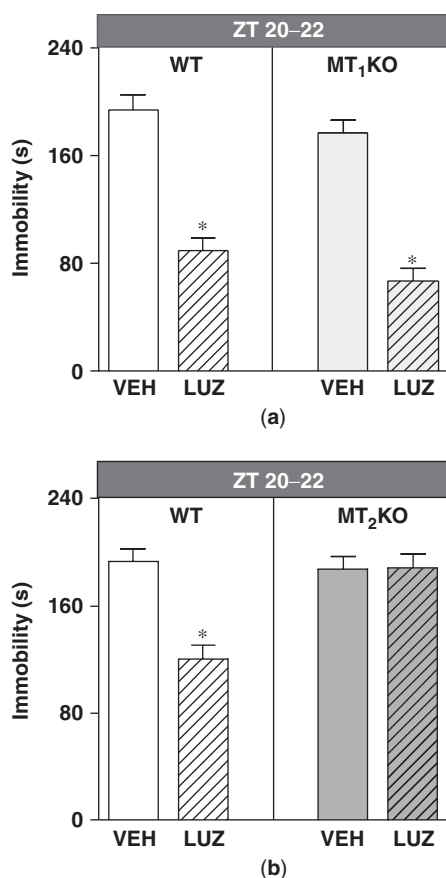


Figure 2.9 Effect of luzindole on swimming test immobility in C3H/HeN WT, MT₁ KO, and MT₂ KO young male mice during dark phase. The ordinate represents total immobility time during 4 min of a 6-min swimming session expressed in seconds. Male mice (2.5 months old) were treated with either vehicle (VEH) or luzindole (LUZ) (30 mg/kg, i.p.) 30 min prior to the forced swimming test. Luzindole treatment during the dark phase (ZT 20–22) significantly decreased time spent immobile in both wild-type (WT) and MT₁ knockout (KO) mice as compared with vehicle-treated [(a) WT and MT₁ KO; (b) WT] but not in the MT₂ KO mice as compared with the corresponding vehicle-treated controls. Bars represent mean \pm SEM of 6–8 (a) and 10 (b) mice per group. (*) $P < 0.001$ as compared with vehicle treated. [Modified (Figs. 1 and 5) from [268] with permission from Blackwell Publishing.]

Agomelatine appears to be superior over other current antidepressant treatments as it promotes sleep [239, 274], entrains circadian rhythms [211, 275], and shows anxiolytic effects and shorter onset of therapeutic effectiveness [269, 270, 272]. It induces dizziness; however, it lacks prominent side effects shown by most classes of antidepressants, such as sexual dysfunction, gastrointestinal reactions, and discontinuation symptoms [269, 272, 276]. Together, these results suggest that agomelatine, with a pharmacokinetic and pharmacological profile distinct from that of other antidepressant, appears to be more effective with faster onset of action and better adverse effect profile than currently prescribed antidepressant medications.

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3

NARCOLEPSY: NEUROPHARMACOLOGICAL ASPECTS

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3.1	Introduction	80
3.2	Symptoms of Narcolepsy	80
3.2.1	Sleepiness or Excessive Daytime Sleepiness	82
3.2.2	Cataplexy	82
3.2.3	Hypnagogic or Hypnopompic Hallucinations	83
3.2.4	Sleep Paralysis	83
3.3	Narcolepsy Evaluation	83
3.3.1	Polysomnography: Nocturnal and Daytime Sleep Studies	83
3.3.2	Cerebrospinal Fluid Hypocretin-1 Assessment	84
3.3.3	Histocompatibility Human Leukocyte Antigen Testing	84
3.4	Idiopathic Hypersomnia and Other Primary EDS	84
3.5	Pathophysiology of Narcolepsy	84
3.5.1	Pathophysiological Consideration of Symptoms of Narcolepsy	84
3.5.2	HLA: Immune System and Narcolepsy	85
3.5.3	Deficiency in Hypocretin (Orexin) Transmission in Canine and Human Narcolepsy	86
3.5.4	Hypocretin /Orexin System and Sleep Regulation	86
3.6	Treatments of Narcolepsy	90
3.6.1	Pharmacological Treatment of EDS with Amphetamine-Like Compounds	90
3.6.1.1	Molecular Targets of Amphetamine Action	93
3.6.1.2	Dopaminergic Neurotransmission and EEG Arousal	94
3.6.1.3	Anatomical Substrates of Dopaminergic Effects	97
3.6.1.4	Adverse Effects of Amphetamine and Amphetamine-Like Compounds	98
3.6.1.5	Drug Interactions with Amphetamine and Amphetamine-Like Compounds	98
3.7	Nonamphetamine Stimulants	100
3.7.1	Modafinil	100
3.7.2	Mazindol	103

3.7.3	Bupropion	103
3.7.4	Selegiline (L-Deprenyl)	104
3.7.5	Caffeine	104
3.8	Future Stimulant Treatments	105
3.9	Pharmacological Treatment of Cataplexy	106
3.9.1	Historical Overview of Antidepressants	106
3.9.2	Tricyclic (First-Generation) Antidepressants	107
3.9.3	Second- to Third-Generation Antidepressants	108
3.9.4	Mechanisms of Action of Tricyclic Anticatatplectics	108
3.9.5	Preferential Involvement of Adrenergic Neurotransmission in Control of Canine Cataplexy	109
3.9.6	Receptor Subtypes Involved in Control of Cataplexy	110
3.9.7	Monoamine Oxidase Inhibitors	111
3.9.8	Sodium Oxybate	112
3.10	Treatment of Sleep Paralysis and Hypnagogic Hallucinations	112
3.11	Treatment of Disturbed Nocturnal Sleep	112
3.12	Future Anticatatplectics	113
3.13	Conclusions	114
	References	115

3.1 INTRODUCTION

Narcolepsy is characterized by excessive daytime sleepiness (EDS), cataplexy, and other dissociated manifestations of rapid-eye-movement (REM) sleep (hypnagogic hallucinations and sleep paralysis). Narcolepsy is currently treated with amphetamine-like central nervous system (CNS) stimulants (for EDS) and antidepressants (for cataplexy). Some other classes of compounds, such as modafinil (a nonamphetamine wake-promoting compound for EDS) and γ -hydroxybutyrate (GHB, a short-acting sedative for EDS/fragmented nighttime sleep and cataplexy), given at night are also employed. The major pathophysiology of human narcolepsy has recently been revealed by the extension of discoveries of narcolepsy genes in animal models: About 90% of human narcolepsy–cataplexy has been found to be hypocretin/orexin ligand deficient. This directly led to the development of new diagnostic tests [i.e., cerebrospinal fluid (CSF) hypocretin measures]. Hypocretin replacement is also likely to be a new therapeutic option for hypocretin-deficient narcolepsy, but this is still not available in humans. In this review, the pharmacological aspects of narcolepsy and related disorders are discussed.

3.2 SYMPTOMS OF NARCOLEPSY

Narcolepsy is a syndrome of unknown etiology (prevalence 1 in 2000 [1, 2]) characterized by EDS that is often profound. About 95% of narcoleptic cases are sporadic, but it also occurs in familial forms. Narcolepsy usually occurs in association with cataplexy and other symptoms and signs, which commonly include hypnagogic hallucinations or hypnopompic hallucinations, sleep paralysis, automatic behavior, and disrupted nocturnal sleep (Table 3.1) [3]. Symptoms most often begin during

TABLE 3.1 Clinical Characteristics of Narcolepsy–Cataplexy, Narcolepsy Without Cataplexy, and Idiopathic Hypersomnia

	Daytime sleepiness		Other Symptoms	MSLT		HLA-DQB1*0602	Low CSF Hypocretin Levels
	Duration	Awaken-Refreshed		Sleep Latency ^a	SOREMPS	Positivity	(<110 pg/mL)
Narcolepsy–cataplexy	Short (<30 min)	(+)	Cataplexy, REM sleep–related symptoms	<8 min	≥2	>90%	85–90% >90% in HLA positive
Narcolepsy without cataplexy	Short (<30 min)	(+)	Cataplexy (–), REM sleep–related symptoms	<8 min	≥2	40–50%	10–20% (almost all HLA positive)
Idiopathic hypersomnia with long sleep time	Long (>30 minutes)	(–)	Cataplexy (–), prolonged nighttime sleep (>10 h), autonomic nervous dysfunction	<8 min	≤1	No consistent results	Normal
Idiopathic hypersomnia without long sleep time	Varied	(–)	Cataplexy (–), no prolonged nighttime sleep (<10 h), autonomic nervous dysfunction	<8 min	≤1	No consistent results	Normal

Abbreviations: MSLT, multiple sleep latency test; HLA, human leukocyte antigen; SOREMP, sudden-onset rapid-eye-movement period.

^aLess than 8 minutes (instead of 5 minutes) was considered for the second revision of the International Classification of Sleep Disorders.

adolescence or young adulthood. However, narcolepsy may also occur earlier in childhood or not until the third or fourth decade of life. Quality-of-life studies suggest that the impact of narcolepsy is equal to that of Parkinson's disease [4]. Although EDS is not specific for narcolepsy and is seen in other primary and secondary EDS disorders (such as sleep apnea syndrome), cataplexy is generally regarded as pathognomonic. Occurrence of cataplexy is tightly associated with loss of hypocretin neurotransmission [5], and it rarely occurs as an isolated symptom. Cataplexy occasionally occurs in conjunction with other neurological conditions, such as Nieman–Pick type C disease, but the pathophysiological links in these neurological conditions with the hypocretin abnormalities are not yet well established [6].

3.2.1 Sleepiness or Excessive Daytime Sleepiness

As with the sleepiness of other sleep disorders, the EDS of narcolepsy presents itself with an increased propensity to fall asleep, nod, or easily doze in relaxed or sedentary situations or a need to exert extra effort to avoid sleeping in these situations [7]. Additionally, irresistible or overwhelming urges to sleep commonly occur from time to time during wakeful periods in the untreated patient with narcolepsy. These so-called sleep attacks are not instantaneous lapses into sleep, as is often thought by the general public, but represent the episodes of profound sleepiness experienced by those with marked sleep deprivation or other severe sleep disorders. In addition to frank sleepiness, the EDS of narcolepsy (as in other sleep disorders) can cause related symptoms, including poor memory, reduced concentration or attention, and irritability. Narcoleptic subjects feel refreshed after a short nap, but this does not last long and they become sleepy again within a few hours (Table 3.1). Narcolepsy may therefore consist of an inability to maintain wakefulness combined with the intrusion of REM sleep–associated phenomena (hypnagogic hallucinations, sleep paralysis, and possibly cataplexy; see below) into wakefulness.

3.2.2 Cataplexy

Cataplexy is the partial or complete loss of bilateral muscle tone in response to a strong emotion [7]. Reduced muscle tone may be minimal, occur in a few muscle groups, and cause minimal symptoms such as bilateral ptosis, head drooping, slurred speech, or dropping things from the hand or may be so severe that total body paralysis occurs, resulting in complete collapse. Cataplectic events usually last from a few seconds to 2 or 3 min but occasionally continue longer [8]. The patient is usually alert and oriented during the event despite the inability to respond. Positive emotions such as laughter more commonly trigger cataplexy than negative emotions; however, any strong emotion is a potential trigger [9]. Startling stimuli, stress, physical fatigue, or sleepiness may also be important triggers or factors that exacerbate cataplexy.

The current international criterion for narcolepsy does not require cataplexy for diagnosing narcolepsy if REM sleep abnormalities (i.e., SOREMPs during MSLT) are objectively documented (Table 3.1). According to epidemiological studies, cataplexy is found in 60–100% of patients with narcolepsy. A large range in the percentage affected with cataplexy is reported because the definitions of narcolepsy vary among studies (and by use of different diagnostic criteria). The onset of cataplexy is most frequently simultaneous with or within a few months of the onset of EDS, but in some cases, cataplexy may not develop until many years after the initial onset of EDS [8].

3.2.3 Hypnagogic or Hypnopompic Hallucinations

These phenomena may be visual, tactile, auditory, or multisensory events that are usually brief but occasionally continue for a few minutes that occur at the transition from wakefulness to sleep (hypnagogic) or from sleep to wakefulness (hypnopompic) [7]. Hallucinations may contain elements of dream sleep and consciousness combined and are often bizarre or disturbing to patients.

3.2.4 Sleep Paralysis

Sleep paralysis is the inability to move, lasting from a few seconds to a few minutes, during the transition from sleep to wakefulness or from wakefulness to sleep [7]. Episodes of sleep paralysis may alarm patients—particularly those who experience the sensation of being unable to breathe. Although accessory respiratory muscles may not be active during these episodes, diaphragmatic activity continues and air exchange remains adequate.

Other commonly reported symptoms include automatic behavior—“absent-minded” behavior or speech that is often nonsensical which the patient does not remember—and fragmented nocturnal sleep—frequent awakenings during the night.

Hypnagogic hallucinations, sleep paralysis, and automatic behavior are not specific to narcolepsy and occur in other sleep disorders (as well as in healthy individuals); however, these symptoms are far more common and occur with much greater frequency in narcolepsy [7].

3.3 NARCOLEPSY EVALUATION

3.3.1 Polysomnography: Nocturnal and Daytime Sleep Studies

Nocturnal polysomnography is not essential in the diagnostic workup when straightforward cataplexy accompanies EDS. However, it remains an important part of the evaluation process primarily to exclude other conditions that occur in narcolepsy at a higher than normal rate (obstructive sleep apnea, periodic limb movement syndrome, and REM sleep behavior disorder) and could add to the sleepiness or nocturnal sleep disruption the patient may be experiencing [10]. Additionally, SOREMPs during nocturnal polysomnography may be witnessed and are also supportive for the diagnosis.

Daytime nap studies (in the form of the MSLT), usually demonstrate substantially reduced sleep latency and SOREMPs in patients with narcolepsy. Average MSLT sleep latencies for narcolepsy with cataplexy are approximately 2–3 min [11]; however, substantial variability across and within patients can, at times, be seen, and mean sleep latencies of less than 5 min during the MSLT are used for the International Classification of Sleep Disorders (ICSD) (less than 8 min was considered in the second revision of the ICSD) (Table 3.1). SOREMPs are also not specific for narcolepsy, but the occurrence of two or more of these events during the MSLT in the setting of objectively marked sleepiness and without any other explanation for their occurrence (such as sleep deprivation, REM-suppressant medication rebound, altered sleep schedule, obstructive sleep apnea, or delayed sleep-phase syndrome) is suggestive of narcolepsy.

3.3.2 Cerebrospinal Fluid Hypocretin-1 Assessment

As discussed in the Section 3.5, many (about 90% of narcolepsy–cataplexy subjects), but not all, patients with narcolepsy have very low or undetectable levels of hypocretin-1/orexin A in the CSF [5, 12]. Such low levels of CSF hypocretin-1 are relatively specific for narcolepsy–cataplexy but are also seen in a few other neurological conditions, such as a subset of patients with Guillain–Barré syndrome and Ma2 positive paraneoplastic syndrome [13, 14]. Since these conditions are clinically distinct from narcolepsy, low CSF hypocretin levels in these conditions do not confound their diagnostic values. When used to assess patients for narcolepsy, low CSF hypocretin-1 appears to be a more specific test than the MSLT and CSF hypocretin-1 levels (Table 3.1). Therefore, low CSF hypocretin-1 levels (less than 110 pg/mL) was included for the second revision of ICSD. Previously, no specific and sensitive diagnostic test for narcolepsy based on the pathophysiology of the disease was available, and the final diagnosis was often delayed for several years after the disease onset, which is typically during adolescence [15]. Many patients with narcolepsy and related EDS disorders are therefore likely to obtain immediate benefit from this new specific diagnostic test.

3.3.3 Histocompatibility Human Leukocyte Antigen Testing

A very strong but incomplete correlation exists between narcolepsy (with cataplexy) and the human leucocyte antigen (HLA) subtype DQB1*0602, yet this subtype is very common in the general population (approximately 20% in the combined U.S. population) and is neither specific nor sensitive for narcolepsy [2]. HLA testing is therefore not useful in confirming or excluding the diagnosis of narcolepsy and, in fact, may lead a clinician to inappropriate diagnostic conclusions (see also Section 3.5).

3.4 IDIOPATHIC HYPERSOMNIA AND OTHER PRIMARY EDS

Less common forms of hypersomnia include the idiopathic and recurrent hypersomnias [16]. Idiopathic hypersomnia is marked by excessive nocturnal sleep of good quality and by EDS which is not as severe as in the narcoleptic patients (but no refreshing after naps) and which is not REM sleep related (Table 3.1). The best characterized recurrent hypersomnia is the Kleine–Levin syndrome (KLS), a pervasive functional disorder of the hypothalamus characterized by hypersexuality, binge eating, and irritability associated with periods of EDS and sleep periods as long as 18–20 h [17, 18]. For details of clinical symptoms and treatments of these primary EDS, refer to [16].

3.5 PATHOPHYSIOLOGY OF NARCOLEPSY

3.5.1 Pathophysiological Consideration of Symptoms of Narcolepsy

The similarity between cataplexy and REM sleep atonia [the presence of frequent episodes of hypnagogic hallucinations and of sleep paralysis and the propensity for narcoleptics to go directly from wakefulness into REM sleep (i.e., SOREMs)]

suggests that narcolepsy is primarily a “disease of REM sleep” [19]. This hypothesis may, however, be too simplistic and does not explain the presence of sleepiness during the day and the short latency to both non-REM (NREM) and REM sleep during nocturnal and nap recordings. Another complementary hypothesis is that narcolepsy results from the disruption of the control mechanisms of both sleep and wakefulness or, in other words, of the vigilance state boundary problems [20]. According to this hypothesis, a cataplectic attack represents an intrusion of REM sleep atonia during wakefulness, while the hypnagogic hallucinations appear as dreamlike imagery taking place in the waking state, especially at sleep onset in patients who frequently have SOREMs.

Cataplexy is associated with an inhibition of the monosynaptic H reflex (a muscle response with monosynaptic latency due to excitation of Ia afferents in the spinal cord) and the polysynaptic deep-tendon reflexes [21]. In control subjects, it is only during REM sleep that the H reflex is totally suppressed. This finding highlights the relationship between the inhibition of motor processes during REM sleep and the sudden atonia and areflexia seen during cataplexy. Studies in canine narcolepsy, however, suggest that the mechanisms for the induction of cataplexy are different from those for REM sleep [22]. Furthermore, an extended human study confirmed that cataplexy correlates much more highly to hypocretin-deficient narcolepsy (see below), in contrast to other REM sleep-related phenomena [5]. Cataplexy may thus be viewed as somewhat distinct from other REM-related symptoms and as a hypocretin-deficiency pathological phenomenon. The fact that patients with other sleep disorders, such as sleep apnea, and even healthy controls can manifest SOREMs, hypnagogic hallucinations, and sleep paralysis when their sleep-wake patterns are sufficiently disturbed yet these subjects never develop cataplexy provides further support to the proposal that cataplexy may be unrelated to other REM-associated symptoms [23–26]. Although cataplexy and REM sleep atonia have great similarity and possibly share a common executive system, it is not necessary for the regulatory mechanism of both states to be identical. The mechanism of emotional triggering of cataplexy remains undetermined.

3.5.2 HLA: Immune System and Narcolepsy

An abundant amount of research into the HLA association in narcolepsy has been conducted which has yielded important discoveries. However, the research has yet to provide clinically meaningful information. This work therefore is only very briefly addressed in this review. A remarkably high HLA association with narcolepsy was discovered in the early 1980s [27]. Since the time of this initial finding, a variety of research across multiple ethnic groups has corroborated the existence of this strong HLA association. The most specific marker of narcolepsy in a number of different ethnic groups studied to date is DQB1*0602 [2]. This association is seen in an average of approximately 90% of those with unequivocal cataplexy [28]. Importantly, this association is substantially lower (only approximately 40%) in individuals who have received the diagnosis of narcolepsy but do not have cataplexy (Table 3.1).

The strong association between HLA type and narcolepsy with cataplexy raises the possibility that narcolepsy is an autoimmune disease [29]. There is, however, no evidence of inflammatory processes or immune abnormalities associated with narcolepsy [29], and studies have found no classical auto-antibodies and no increase in oligoclonal CSF bands in narcoleptics [30]. Typical autoimmune pathologies

(erythrocyte sedimentation rates, serum immunoglobulin levels, C-reactive protein levels, complement levels, and lymphocyte subset ratios) are apparently normal in narcoleptic patients [31]. In contrast, a variety of serological tests performed in narcoleptics, along with age- and sex-matched controls, yielded higher levels of antistreptolysin 0 and anti-DNase antibodies in narcoleptics than in controls [32, 33]. In view of these preliminary data, further exploration of possible immune-related dysfunction in narcolepsy is warranted.

3.5.3 Deficiency in Hypocretin (Orexin) Transmission in Canine and Human Narcolepsy

Narcolepsy has been described in several animal species, including dogs and most recently genetically engineered mice and rat models. Canine narcolepsy is a naturally occurring model, with both sporadic (17 breeds) and familial forms (Doberman, Labrador, and Dachshund). In Doberman pinschers and Labrador retrievers, the disease is transmitted as a recessive autosomal trait with complete penetrance [34].

In 1999, using positional cloning and gene-targeting strategies, two groups independently revealed the pathogenesis of narcolepsy in animals. The lack of the hypothalamic neuropeptide hypocretin/orexin ligand [preprohypocretin/orexin gene knockout (KO) mice] [35] or mutations in one of the two hypocretin/orexin receptor genes [hypocretin receptor 2 (*Hcrtr2*) gene in autosomal recessive canine narcolepsy] [36] was observed to result in narcolepsy. After extensive screening (especially in familial and early-onset human narcolepsy), it was demonstrated that mutations in hypocretin-related genes are rare: Only a single case with early-onset (six months of age) was found to be associated with a single point mutation in the preprohypocretin gene [37]. This result was however not surprising considering the fact that a large majority of human narcolepsy cases are sporadic. Even in rare familial cases of narcolepsy, it is unlikely that high-penetrant single genes (like hypocretin related genes) are involved.

Despite the lack of genetic abnormalities in the hypocretin system, it was found that the large majority (85–90%) of patients with narcolepsy–cataplexy have low or undetectable hypocretin-1 ligand in their CSF [12, 38] (Fig. 3.1). This hypocretin deficiency is tightly associated with the occurrence of cataplexy and HLA-DQB1*0602 positivity [5, 39, 40]. Postmortem human studies (utilizing a few brains) have confirmed hypocretin ligand deficiency (both hypocretin 1 and 2) in the narcoleptic brain [37, 41] (Fig. 3.1). Hypocretin deficiency has also been observed in sporadic cases of canine narcolepsy (seven of seven currently studied; the result of four cases are reported), suggesting that the pathophysiology in these animals mirrors that of most human cases [42].

Although the etiology of hypocretin deficiency in humans still needs to be determined, the fact that a large majority of human narcolepsy–cataplexy subjects are hypocretin ligand deficient suggests that hypocretin agonists may be promising in the treatment of narcolepsy (see Section 3.8).

3.5.4 Hypocretin /Orexin System and Sleep Regulation

Hypocretins/orexins were only recently discovered (in 1998, one year prior to the cloning of the canine narcolepsy gene) by two independent research groups. One

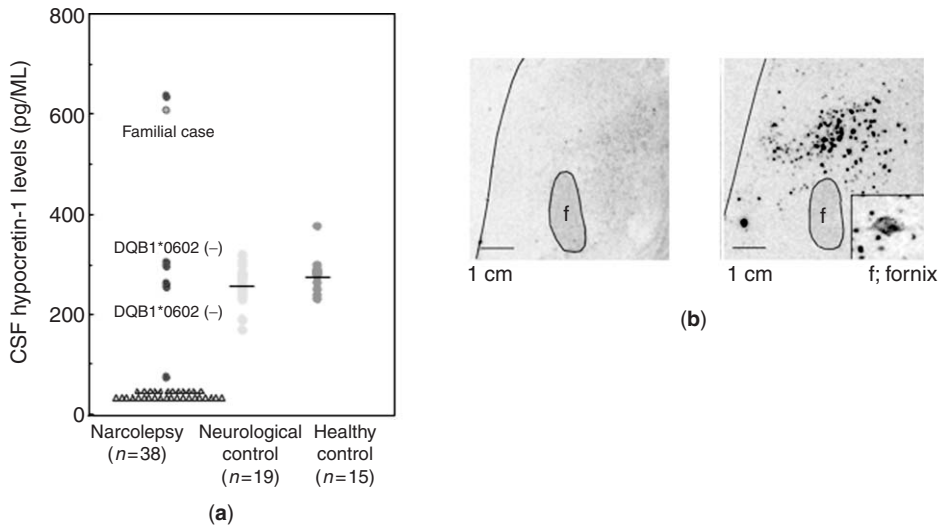


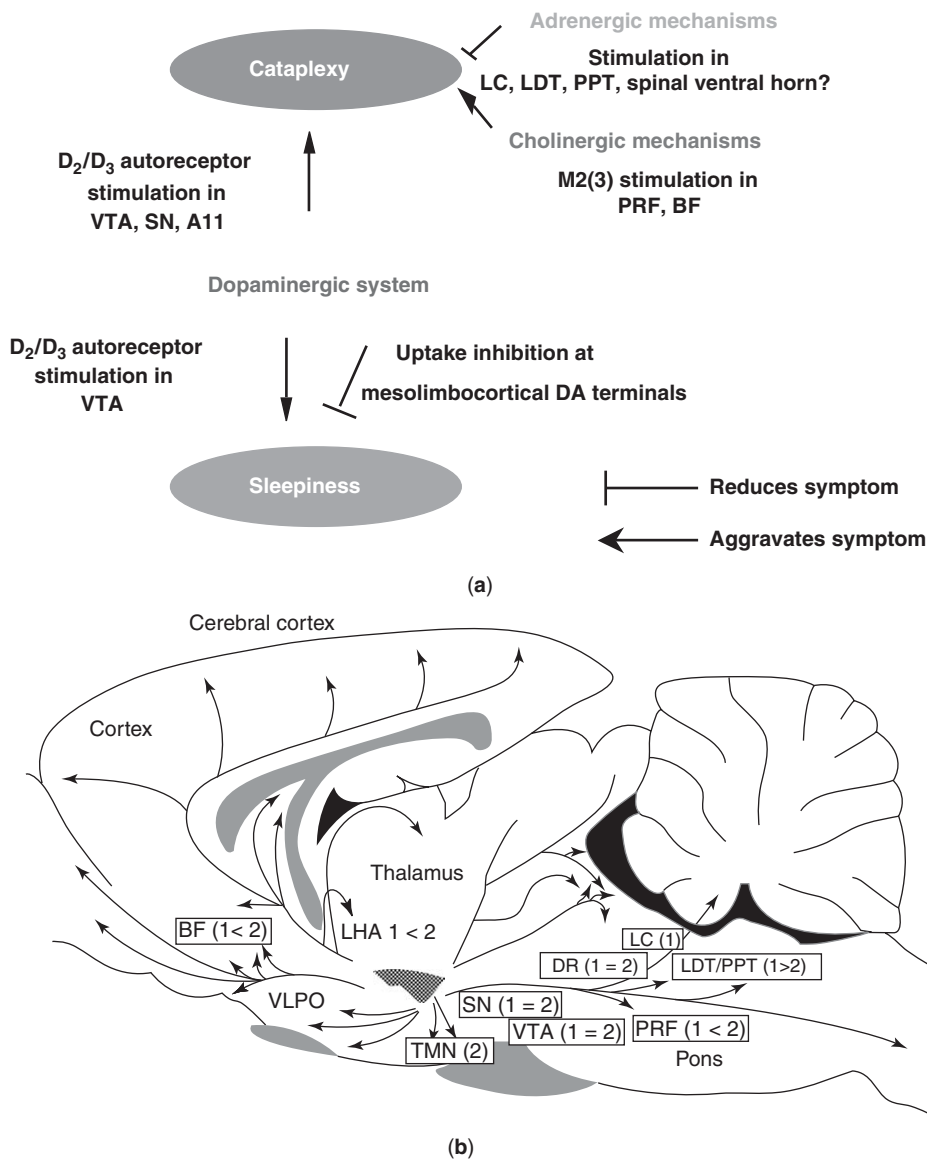
Figure 3.1 (a) CSF hypocretin-1 levels in narcoleptic and control subjects. CSF hypocretin-1 levels are undetectably low in most narcoleptic subjects (84.2%). Note that two HLA DQB1*0602 negative and one familial case have normal or high CSF hypocretin levels. (b) Preprohypocretin mRNA in situ hybridization in the hypothalamus of control and narcoleptic subjects. Preprohypocretin transcripts are detected in the hypothalamus of control (right) but not narcoleptic (left) subjects. Melanin-concentrating hormone (MCH) transcripts are detected in the same region in control and narcoleptic sections (data not shown). (See color insert.)

group called the peptides “hypocretin” because of their primary hypothalamic localization and similarities with the hormone secretin [43]. The other group called the molecules “orexin” after observing that central administration of these peptides increased appetite in rats [44]. Hypocretins 1 and 2 are produced exclusively by a well-defined group of neurons localized in the lateral hypothalamus. The neurons project to the olfactory bulb, cerebral cortex, thalamus, hypothalamus and brain stem, particularly the locus ceruleus (LC), raphe nucleus, and cholinergic nuclei (the laterodorsal tegmental and pedunculopontine tegmental nuclei), and cholinceptive sites (such as pontine reticular formation) thought to be important for sleep regulation [45] (Fig. 3.2).

A series of recent studies have now shown that the hypocretin system is a major excitatory system that affects the activity of monoaminergic [dopamine (DA), norepinephrine (NE), serotonin (5-HT), and histamine] and cholinergic systems with major effects on vigilance states [46, 47] (Fig. 3.2). It is thus likely that a deficiency in hypocretin neurotransmission induces an imbalance between these classical neurotransmitter systems, with primary effects on sleep state organization and vigilance (Fig. 3.2). Indeed, DA and/or NE contents have been reported to be high in several brain structures in narcoleptic Dobermans and in human narcolepsy brains postmortem [48]. These changes are possibly due to compensatory mechanisms, since the drugs that enhance dopaminergic neurotransmission [such as amphetamine-like stimulants and modafinil (for EDS)] and NE neurotransmission [such as noradrenaline uptake blockers (for cataplexy)] are needed to subside the symptoms of narcolepsy [48]. Histamine is another monoamine implicated in the

control of vigilance, and the histaminergic system is also likely to indirectly mediate the wake-promoting effects of hypocretin [49–51]. Interestingly, brain histamine contents both in *hcrtr2* gene mutated and ligand-deficient narcoleptic dogs are dramatically reduced [52]. It is thus interesting to further evaluate the involvement of the histaminergic system in the pathophysiology of narcolepsy and therapeutic applications of histaminergic compounds [53].

Many measurable activities (brain and body) and compounds manifest rhythmic fluctuations over the 24-h period. Whether hypocretin tone changes with *zeitgeber* time was assessed by measuring extracellular hypocretin-1 levels in the rat brain CSF



across 24-h periods using *in vivo* dialysis [54–56]. The results demonstrate the involvement of a slow diurnal pattern of hypocretin neurotransmission regulation (as in the homeostatic and/or circadian regulation of sleep). Hypocretin levels increase during the active periods and are highest at the end of the active period, and the levels decline with the onset of sleep. Furthermore, sleep deprivation increases hypocretin levels [54–56].

Changes in firing of hypocretin neuronal across different sleep stages were recently reported in rats; hypocretin neurons are wake-active and their activities reduce during slow wave sleep (SWS) and REM sleep [56a,b]. Regardless of the firing pattern of the hypocretin neurons, our results however suggest that basic hypocretin neurotransmission fluctuates across the 24-h period and slowly builds up during the active period. Adrenergic LC neurons are typical wake-active neurons involved in vigilance control, and it has been recently demonstrated that basic firing activity of wake-active LC neurons also significantly fluctuates across various circadian times [57].

Several acute manipulations such as exercise, low glucose utilization in the brain, and forced wakefulness increase hypocretin levels [46, 55, 58]. It is therefore hypothesized that a buildup/acute increase of hypocretin levels may counteract homeostatic sleep propensity that typically increases during the daytime and during forced wakefulness [55]. Due to the lack of increase in hypocretin tone, narcoleptic subjects may not be able to stay awake for a prolonged period and do not respond to various alerting stimuli. Conversely, reduction of the hypocretin tone at sleep onset may contribute to the profound deep sleep that normally inhibits REM sleep at sleep onset, and the lack of this system in narcolepsy may allow the occurrence of REM sleep at sleep onset.

Figure 3.2 (a) Monoaminergic and cholinergic control of sleepiness and cataplexy in relation to hypocretin input: schematic perspective. The stimulation of adrenergic transmission by adrenergic uptake inhibitors potently reduces cataplexy; this pharmacological property is likely involved in the mode of action of currently used anticataplectic agents (e.g., tricyclic antidepressants). The fact that both presynaptic α_2 autoreceptor stimulation and postsynaptic α_1 blockade aggravate cataplexy is consistent with an inhibitory role of adrenergic transmission in the control of REM sleep atonia. Dopaminergic uptake inhibitors have no effect on cataplexy, although these compounds strongly induce electrocortical arousal. In contrast, D_2/D_3 autoreceptor stimulation aggravates both cataplexy and sleepiness. Since DA uptake inhibitors are reported to be mostly active at the level of mesocortical and mesolimbic DA terminals, DA projections to these regions may be more involved in mediating EEG arousal. Muscarinic M_2 stimulation induces behavioral wakefulness and cortical desynchrony in control dogs, while it induces cataplexy in narcoleptic dogs. Although muscarinic antagonists reduce cataplexy in the canine model, attempts to use this class of compounds in humans have not been successful mainly due to the side effects. (b) Projections of hypocretin neurons in the rat brain and relative abundances of hypocretin receptor 1 and 2. It was recently revealed that hypocretin-containing neurons project to these previously identified monoaminergic and cholinergic and cholinceptive regions where hypocretin receptors are enriched. Impairments of hypocretin input may thus result in cholinergic and monoaminergic imbalance and generation of narcoleptic symptoms. VTA, ventral tegmental area; SN, substantia nigra; LC, locus ceruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; BF, basal forebrain; VLPO, ventrolateral preoptic nucleus; LHA, lateral hypothalamic area; TMN; tuberomammillary nucleus; DR, dorsal raphe. (See color insert.)

3.6 TREATMENTS OF NARCOLEPSY

Nonpharmacological treatments (i.e., by behavioral modification) are often reported to be useful additions to the clinical management of narcoleptic patients [59–62]. Regular napping usually relieves sleepiness for 1–2 h [59] and is the treatment of choice for some patients, but this often has negative social and professional consequences. Exercising to avoid obesity, keeping a regular sleep–wake schedule, and having a supportive social environment (e.g., patient group organizations and support groups) are also helpful. In almost all cases, however, pharmacological treatment is needed, and 94% of patients reported using medications in a recent survey by a patient group organization [63].

3.6.1 Pharmacological Treatment of EDS with Amphetamine-Like Compounds

Amphetamine was first synthesized in 1897, but its stimulant effect was only recognized in 1929 by Alles. In 1935, amphetamine was used for the first time for the treatment of narcolepsy, and this was possibly the first condition for which amphetamine was used clinically, and it also revolutionized therapy for the condition (even though it was not a curative). The piperazine derivative of amphetamine, methylphenidate, was introduced for the treatment of narcolepsy in 1959 by Yoss and Daly [64], but both compounds share similar pharmacological properties.

Amphetamine and methylphenidate are primarily indicated for narcolepsy (Table 3.2), idiopathic hypersomnia, and attention-deficit hyperactivity disorder (ADHD). However, other therapeutic uses are controversial because of their abuse potential. As a result, amphetamine is classified as a Schedule II substance and

TABLE 3.2 Current Pharmacological Treatment for Human Narcolepsy and Its Related Disorders

Compound	Usual Daily Doses (mg)	Half-Life (h)	Side Effects/Notes
Wake-promoting compounds:			
Sympathomimetic stimulants: d-amphetamine sulfate	5–60	16–30	Irritability, mood changes, headaches, palpitations, tremors, excessive sweating, insomnia
Methylphenidate HCl	10–60	~3	Same as amphetamines, less reduction of appetite or increase in blood pressure
Pemoline	20–115	11–13	Less sympathomimetic effect, milder stimulant, slower onset of action, occasionally produces liver toxicity
Nonamphetamine wake-promoting compounds: modafinil	100–400	11–14	No peripheral sympathomimetic action, headache, nausea

methylphenidate is classified as a Schedule III substance under the Controlled Substances Act of 1970.

Phenylisopropylamine (amphetamine) has a simple chemical structure resembling endogenous catecholamines (Fig. 3.3). The pharmacological effects of most amphetamine derivatives are isomer specific. D-Amphetamine, for example, is a far more potent stimulant than the L-derivative. In EEG studies, D-amphetamine is four times more potent in inducing wakefulness than L-amphetamine [65]; not all effects are, however, specific. For example, both enantiomers are equipotent at suppressing REM sleep in humans and rats [65] and at producing amphetamine psychosis. The relative effects of the D- and L- isomers of amphetamine on NE and DA transmission may explain some of these differences (for details, see Section 3.6.1.2).

Amphetamine-like compounds, such as methylphenidate, pemoline, and fencamfamin, are structurally similar to amphetamines; all compounds include a benzene core with an ethylamine group side chain (Fig. 3.3). Both methylphenidate and pemoline were commonly used for the treatment of EDS in narcolepsy, but pemoline has been withdrawn from the market in several countries because of liver toxicity (see Table 3.2). The most commonly used commercially available form of methylphenidate is a racemic mixture of both the D- and L- enantiomers, but D-methylphenidate

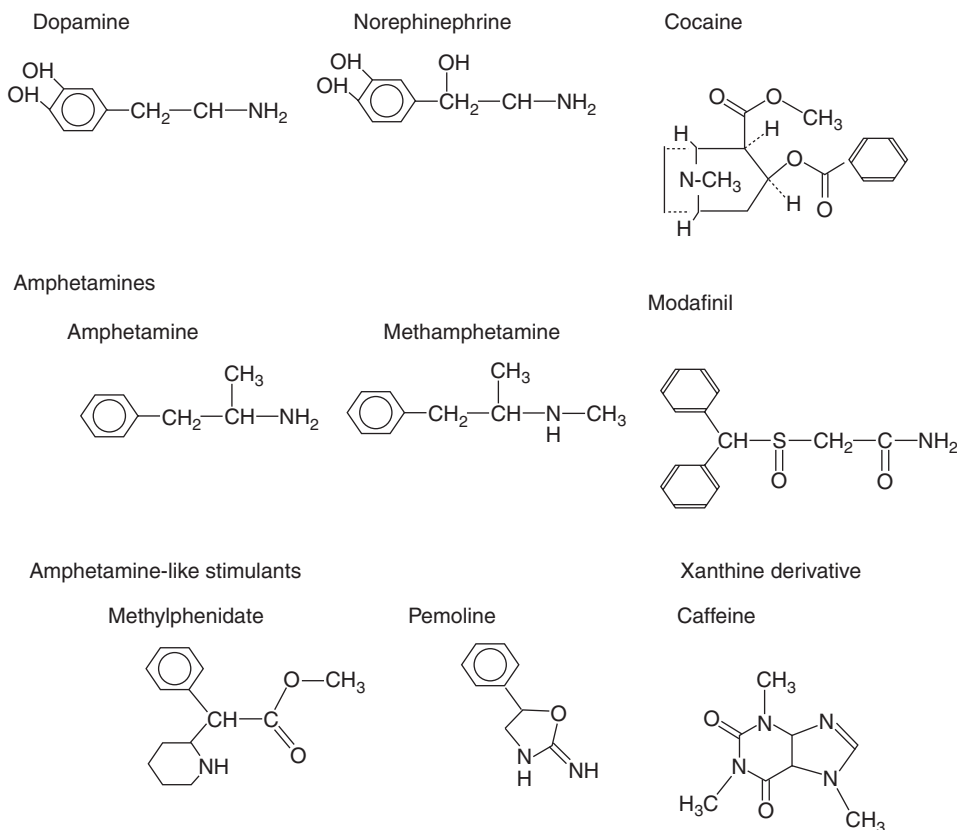
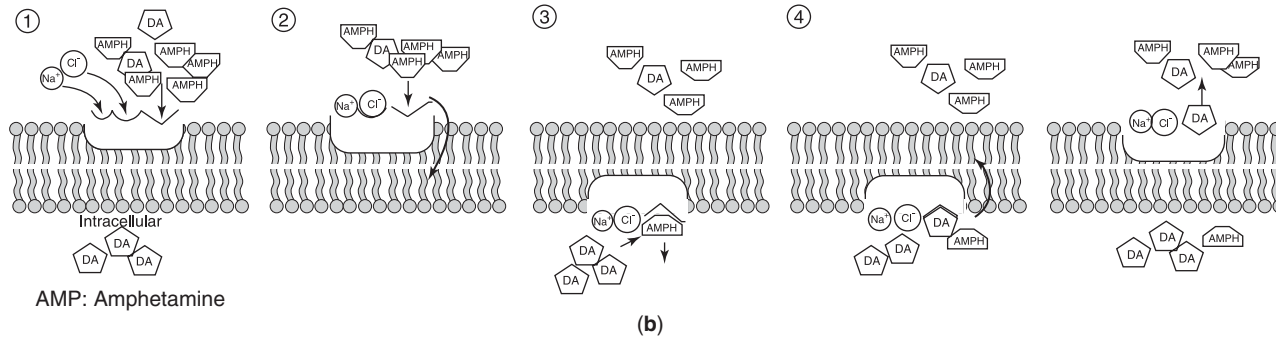
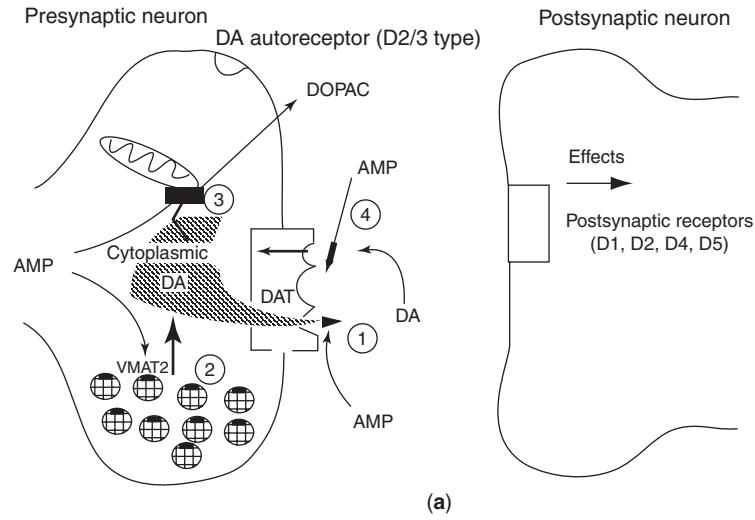


Figure 3.3 Chemical structures of amphetamine-like stimulants modafinil and caffeine (a xanthine derivative) as compared to DA and NE. (See color insert.)



mainly contributes to clinical effects, especially after oral administration. This is due to the fact that L-methylphenidate, but not D-methylphenidate, undergoes a significant first-pass metabolism (by deesterification to L-ritalinic acid).

Amphetamines are highly lipid soluble molecules that are well absorbed by the gastrointestinal tract. Peak levels are achieved approximately 2 h after oral administration, with rapid tissue distribution and brain penetration. Protein binding is highly variable, with an average volume of distribution (V_d) of 5 L/kg. The effects of amphetamine last approximately 6–10 h, with a half-life of 16–30 h. Both hepatic catabolism and renal excretion are involved in the inactivation of amphetamine. Methylphenidate is almost totally absorbed after oral administration, and peak levels are achieved approximately within 1–2 h, with rapid tissue distribution and brain penetration; slow release forms (peak levels of 5–6 h) are also available. Methylphenidate has low protein binding (15%) and is fairly short acting; the effects last approximately 4 h, with a half-life of 3 h. The primary means of clearance is through the urine, in which 90% is excreted.

3.6.1.1 Molecular Targets of Amphetamine Action. The molecular targets mediating amphetamine-like stimulant effects are complex and vary depending of the specific analog/isomer and the dose administered. Amphetamine per se increases catecholamine (DA and NE) release and inhibits reuptake. These effects are mediated by specific catecholamine transporters [66] (Fig. 3.4). The DA transporter (DAT) and the NE transporter (NET) have now been cloned and characterized. The DAT and NET proteins are about 620-amino-acid proteins with 12 putative membrane-spanning regions. Amphetamine derivatives inhibit the uptake and enhance the release of DA, NE, or both by interacting with these molecules. The DAT and NET normally move DA and NE, respectively, from the outside to the inside of the cell. This process is sodium dependent; sodium and chloride bind to the DA/NE transporter to immobilize it at the extracellular surface and to alter the conformation of the DA/NE binding site, thereby facilitating substrate binding. Substrate binding allows movement of the carrier to the intracellular surface of the neuronal membrane, driven by sodium concentration gradients. Interestingly, in the presence

Figure 3.4 (a) Effects of amphetamines at dopaminergic nerve terminal. (1) Amphetamine interacts with the DAT carrier to facilitate DA release from the cytoplasm through an exchange diffusion mechanism. At higher intracellular concentrations, amphetamine also (2) disrupts vesicular storage of DA and (3) inhibits the monoamine oxidase (MAO). Both these actions increase cytoplasmic DA concentrations. (4) Amphetamine also inhibits DA uptake by virtue of its binding to and transport by the DAT; DOPAC, dihydroxyphenylacetic acid. (b) Schematic model of exchange-diffusion process in relation to mode of action of amphetamines. (1) Sodium and chloride bind to the DAT to immobilize it at the extracellular surface. This alters the conformation of the DA binding site on the DAT to facilitate substrate binding. (2) Amphetamine, in competition with extracellular DA, binds to the transporter. Substrate binding allows the movement of the carrier to the intracellular surface of the neuronal membrane, driven by the sodium and amphetamine concentration gradients, resulting in a reversal of the flow of DA uptake. (3) Amphetamine dissociates from the transporter, making the binding site available to cytoplasmic DA. (4) DA binding to the transporter enables the movement of the transporter to the extracellular surface of the neuronal membrane, as driven by the favorable DA concentration gradient. (5) DA dissociates from the transporter, making the transporter available for amphetamine, and thus another cycle. (Adapted from [2].)

of some drugs such as amphetamine, the direction of transport appears to be reversed (Fig. 3.4). DA and NE are moved from the inside of the cell to the outside through a mechanism called exchange diffusion, which occurs at low doses (1–5 mg/kg) of amphetamine, and this mechanism is involved in the enhancement of catecholamine release by amphetamine. A recent in vitro experiment has shown that amphetamine transportation causes an inward current, and intracellular sodium ion becomes more available, thereby enhancing DAT-mediated reverse transport of DA.

At higher doses, other effects are involved. Moderate to high doses of amphetamine (> 5 mg/kg) interact with the vascular monoamine transporter 2 (VMAT2) (see [66]). The vesicularization of the monoamines (DA, NE, 5-HT, and histamine) in the central nervous system is dependent on VMAT2, and the VMAT2 regulates the size of the vesicular and cytosolic monoamine pools. Amphetamine is highly lipophilic and easily enters nerve terminals by diffusing across several mechanisms. It leads to a diffusion of the native monoamines out of the vesicles into the cytoplasm along a concentration gradient, and it acts as a physiological VMAT2 antagonist that releases the vascular DA/NE into the cytoplasm. These mechanisms, as well as the reverse transport and the blocking of reuptake of DA/NE by amphetamine, all lead to an increase in NE and DA synaptic concentrations (see [66]). High doses (higher than a clinical dose) of amphetamines are also shown to inhibit monoamine oxidase (MAO) and prevent catecholamine metabolism.

Various amphetamine derivatives have slightly different effects on all these systems. For example, methylphenidate also binds to the NET and DAT and weakly enhances catecholamine release but has less effect on the VMAT granular storage site than amphetamine. Similarly, D-amphetamine has proportionally more releasing effect on the DA versus the NE system when compared to L-amphetamine. Of note, other antidepressant medications acting on catecholamines, including both DA and NE (e.g., bupropion or mazindol), tend to exert their actions by simply blocking the reuptake mechanism.

3.6.1.2 Dopaminergic Neurotransmission and EEG Arousal. How amphetamines and other stimulants increase electroencephalogram (EEG) arousal has been explored using a canine model of the sleep disorder narcolepsy and DAT KO mice models. Canine narcolepsy is a naturally occurring animal model of the human disorder [7]. Similar to human patients, narcoleptic dogs are excessively sleepy (i.e., shorter sleep latency), have fragmented sleep patterns, and display cataplexy [7]. Although amphetamine-like compounds are well known to stimulate catecholaminergic transmission, the exact mechanism by which they promote EEG arousal is still uncertain. Stimulation of either or both adrenergic or dopaminergic transmission has been suggested to play a role.

In order to address this question, the effects of ligands specific for the DA (GBR12909, bupropion, and amineptine), NE (nisoxetine and desipramine), or both DA and NE (mazindol and nomifensine) transporters as well as amphetamine and a nonamphetamine stimulant, modafinil, were studied in narcoleptic and control Dobermans [67] (Fig. 3.5). DA uptake inhibitors such as GBR12909 and bupropion dose dependently increased EEG arousal in narcoleptic dogs, while nisoxetine and desipramine (two potent NE uptake inhibitors) had no effect on EEG arousal at doses which almost completely suppressed REM sleep and cataplexy (see [67]). Most strikingly, the EEG arousal potency of various DA uptake inhibitors correlated

tightly with in vitro DAT binding affinities (Fig. 3.5), while a reduction in REM sleep correlated with in vitro NET binding affinities [67]. These results strongly suggest that DA uptake inhibition is critical for the EEG arousal effects of these compounds.

Of note, D-amphetamine has a relatively low DAT binding affinity but potently (i.e., need for a low milligram-per-kilogram dose) promotes alertness (Fig. 3.5). It is also generally considered to be more efficacious (i.e., can produce more alertness at a higher dose) than pure DAT reuptake inhibitors in promoting wakefulness. However, as described earlier, D-amphetamine not only inhibits DA reuptake but also enhances DA release (at lower dose by exchange diffusion and at higher dose by antagonistic action against VMAT2) and inhibits monoamine oxidation to prevent DA metabolism. The DA releasing effects of amphetamine are likely to explain the unusually high potency and efficacy of amphetamine in promoting EEG arousal.

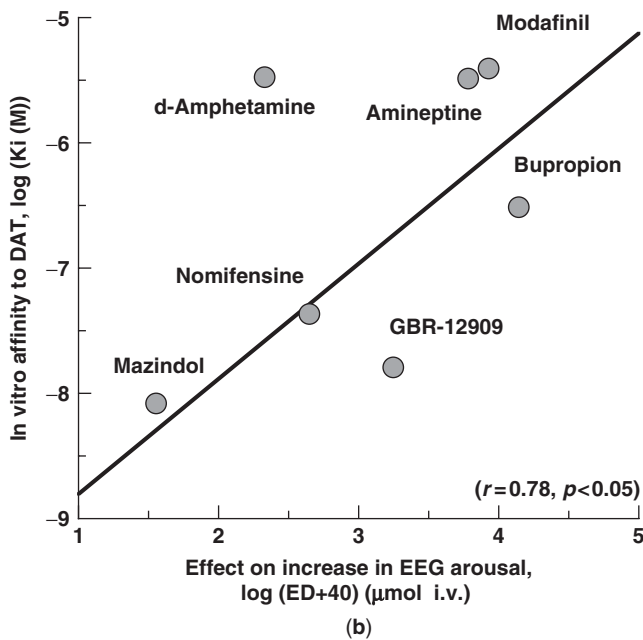
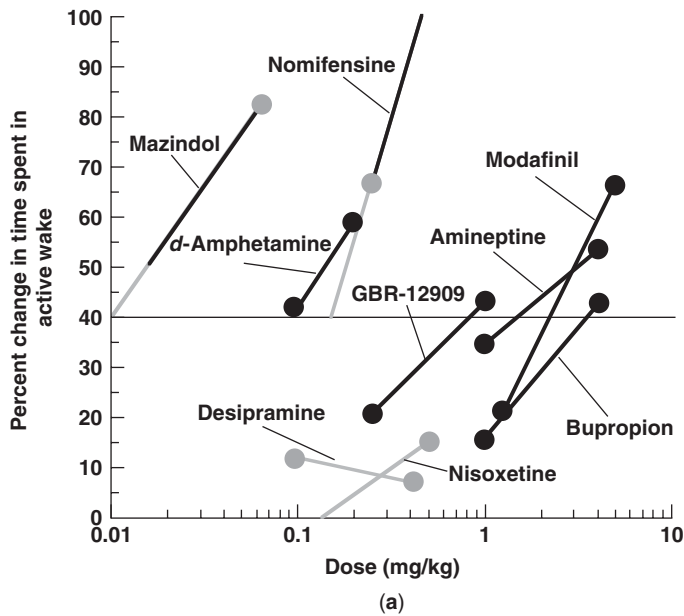
The effects of various amphetamine analogs (D-amphetamine, L-amphetamine, and L-methamphetamine) on EEG arousal and their in vivo effects on brain extracellular DA levels in narcoleptic dogs were compared [68] in order to further differentiate between the involvement of the DA and NE systems in the mode of action of amphetamine derivatives. In vitro studies have demonstrated that the potency and selectivity for enhancing release or inhibiting uptake of DA and NE vary between amphetamine analogs and isomers [69]. Amphetamine derivatives thus offer a unique opportunity to study the pharmacological control of alertness in vivo. Hartmann and Cravens previously reported that D-amphetamine is 4 times more potent in inducing EEG arousal than is L-amphetamine but that both enantiomers are equipotent at suppressing REM sleep in humans and rats [65]. Enantiomer-specific effects have also been reported with methamphetamine; L-methamphetamine is much less potent as a stimulant than either D-methamphetamine or D- or L-amphetamine (see [69]). Similarly, in canine narcolepsy, D-amphetamine is 3 times more potent than L-amphetamine and 12 times more potent than L-methamphetamine in increasing wakefulness and reducing SWS [68].

To further study what mediates these differences in potency, the effects of these amphetamine derivatives on DA release were examined in freely moving animals using in vivo microdialysis. Amphetamine derivatives (100 μ M) were perfused locally for 60 min through the dialysis probe implanted in the caudate of narcoleptic dogs [68]. The local perfusion of D-amphetamine raised DA levels 9 times above baseline. L-Amphetamine also increased DA levels by up to 7 times, but peak DA release was only obtained at the end of the 60-min perfusion period. L-Methamphetamine did not change DA levels under these conditions. These results suggest that D-amphetamine is more potent than L-amphetamine in increasing caudate DA levels, while L-methamphetamine had the least effect—in agreement with data obtained in other species using the same technique [69]. NE was also measured in the frontal cortex during perfusion of D-amphetamine, L-amphetamine, and L-methamphetamine. Although all compounds increased NE efflux, no significant difference in potency was detected among the three analogs.

The fact that the potency of amphetamine derivatives on EEG arousal correlates with effects on DA efflux in the caudate of narcoleptic dogs further suggests that the enhancement of DA transmission by presynaptic modulation mediates the wake-promoting effects of amphetamine analogs. This result is also consistent with data obtained with DAT blockers (see Fig. 3.5). Considering the fact that other amphetamine-like stimulants (such as methylphenidate and pemoline) also inhibit

DA uptake and enhance release of DA, the presynaptic enhancement of DA transmission is likely to be the key pharmacological property mediating wake promotion for all amphetamines and amphetamine-like stimulants.

The role of the DA system in sleep regulation was further assessed using mice lacking the DAT gene. Consistent with a role of DA in the regulation of wakefulness, these animals have reduced non-REM sleep time and increased wakefulness



consolidation (independently from locomotor effects) [70]. DAT KO mice have also proven to be a powerful tool to help dissect the molecular mechanisms mediating the effects of nonselective monoaminergic compounds. Using these animals, DAT was shown to be involved in mediating locomotor activation after amphetamine and cocaine administration. Indeed, no locomotor stimulation is observed in these mice after cocaine or amphetamine. Interestingly, NET KO mice are more sensitive to the locomotor stimulation of amphetamine, suggesting that NET may play a feedback control role on amphetamine-induced dopaminergic effects [71]. With regard to sleep, the most striking finding was that DAT KO mice were completely unresponsive to the wake-promoting effects of methamphetamine, GBR12909 (a selective DAT blocker), and modafinil. These results further confirm the critical role of DAT in mediating the wake-promoting effects of amphetamines and modafinil (Fig. 3.5) [70] (see also Section 3.7.1). Interestingly, DAT KO animals were also found to be more sensitive to caffeine [70], suggesting functional interactions between adenosine and DA systems in the control of sleep/wakefulness (see also Section 3.7.5).

3.6.1.3 Anatomical Substrates of Dopaminergic Effects. Anatomical studies have demonstrated two major subdivisions of the ascending DA projections from mesencephalic DA nuclei (VTA, SN, and retrorubral [A8]): (1) The mesostriatal system originates in the SN and retrorubral nucleus and terminates in the dorsal striatum (principally the caudate and putamen) [72]. (2) The mesolimbocortical DA system consists of the mesocortical and mesolimbic DA systems. The mesocortical

Figure 3.5 (a) Effects of various DA and NE uptake inhibitors and amphetamine-like stimulants on EEG arousal of narcoleptic dogs. The effects of various compounds on daytime sleepiness was studied using 4-h daytime polygraphic recordings (10:00 to 14:00) in four to five narcoleptic animals. Two doses were studied for each compound. All DA uptake inhibitors and CNS stimulants dose-dependently increased EEG arousal and reduced slow-wave sleep (SWS) when compared to vehicle treatment. In contrast, nisoxetine and desipramine, two potent NE uptake inhibitors, had no significant effect on EEG arousal at doses that completely suppressed cataplexy. Compounds with both adrenergic and dopaminergic effects (nomifensine, mazindol, D-amphetamine) were active on both EEG arousal and cataplexy. The effects of the two doses studied for each stimulant were used to approximate a dose–response curve; the drug dose that increased the time spent in wakefulness by 40% above baseline (vehicle session) was estimated for each compound. The order of potency of the compounds obtained was mazindol > (amphetamine) > nomifensine > GBR12 909 > amineptine > (modafinil) > bupropion. (b) Correlation between in vivo EEG arousal effects and in vitro DA transporter binding affinities. In vitro DAT binding was performed using [³H]-WIN 35,428 onto canine caudate membranes. Affinity for the various DA uptake inhibitors tested varied widely between 6.5 and 3.3 mM. In addition, it was also found that both amphetamine and modafinil have a low but significant affinity (same range as amineptine) for the DAT. A significant correlation between in vivo and in vitro effects was observed for all five DA uptake inhibitors and modafinil. Amphetamine, which had potent EEG arousal effects, has a relatively low DAT binding affinity, suggesting that other mechanisms, most probably monoamine-releasing effects or monoamine oxidase inhibition, are also involved. In contrast, there was no significant correlation between in vivo EEG arousal effects and in vitro NE transporter binding affinities for DA and NE uptake inhibitors. These results suggest that presynaptic enhancement of DA transmission is the key pharmacological property mediating the EEG arousal effects of most wake-promoting CNS stimulants. (Adapted from [67].)

system originates in the VTA and the medial SN and terminates in the limbic cortex (medial prefrontal, anterior cingulate, and entorhinal cortices). Interestingly, DA reuptake is of physiological importance for the elimination of DA in cortical hemispheres, limbic forebrain, and striatum but not in midbrain DA neurons. It is thus possible that DA uptake inhibitors (and amphetamine and modafinil) act mostly on DA terminals of the cortical hemispheres, limbic forebrain, and striatum to induce wakefulness. Local perfusion experiments of DA compounds in rats and canine narcolepsy have suggested that the VTA, but not the SN, is critically involved in the regulation of EEG arousal [73]. DA terminals of the mesolimbocortical DA system may thus be important in mediating wakefulness after DA-related CNS stimulant coadministration.

3.6.1.4 Adverse Effects of Amphetamine and Amphetamine-Like Compounds. As described repeatedly, amphetamine releases not only DA but also NE. NE indirectly stimulates α - and β -adrenergic receptors, a profile common to all indirectly acting sympathomimetic compounds. This results in significant cardiovascular effects. α -Adrenergic stimulation produces vasoconstriction, thereby increasing both systolic and diastolic blood pressure. Heart rate may slow down slightly in reflex (this effect is more pronounced than indirect β -adrenergic stimulation on heart rate at low dose). Smooth muscles respond to amphetamine as they do with other sympathomimetic drugs. There is a contractile effect on the urinary bladder sphincter—an effect that has been used in treating enuresis and incontinence.

Other acute side effects include mild gastrointestinal disturbance, anorexia, dryness of the mouth, tachycardia, cardiac arrhythmias, insomnia, restlessness, headaches, palpitations, dizziness, and vasomotor disturbances. Also, agitation, confusion, dysphoria, apprehension, and delirium may occur. Other documented side effects include flushing, pallor, excessive sweating, and muscular pains. Tiredness and sleepiness as well as lethargy and listlessness may also occur when the effects wear off (in addition to a mild mood depression). For common side effects of CNS stimulant drugs in narcoleptics, refer to Table 3.2.

Common side effects occurring during long-term treatment in narcolepsy include irritability, headache, ill temper, and profuse sweating (reported by over one-third of subjects). Other less common side effects include anorexia, gastric discomfort, nausea, talkativeness, insomnia, orofacial dyskinesia, nervousness, palpitations, muscle jerking, chorea, and tremor. Psychiatric symptoms such as delusions or hallucinations may also occur but are rather rare in narcoleptic patients that receive amphetamine. Methamphetamine is more frequently associated with central nervous system complications because of its higher toxicity and central penetrance.

The side-effect profile of methylphenidate is similar to that of amphetamine but is less severe and includes nervousness, insomnia, and anorexia as well as dose-related systemic effects such as increased heart rate and blood pressure. Methylphenidate overdose may lead to seizures, dysrhythmias, and hyperthermia.

3.6.1.5 Drug Interactions with Amphetamine and Amphetamine-Like Compounds. Drug interactions with amphetamine and methylphenidate are generally pharmacodynamic/neurochemical in nature. A small portion of the metabolism of amphetamine and methylphenidate occurs via the cytochrome P450 2D6, and drugs that inhibit 2D6 metabolism can theoretically increase plasma levels of amphetamine.

TABLE 3.3 Antidepressants Currently Used as Anticatataptic Agents

Antidepressants			
Compound	Usual Daily Doses (mg)	Half-life (h)	Notes/Side Effects
Tricyclics			
Imipramine	10–100	5–30	Dry mouth, anorexia, sweating, constipation, drowsiness
Desipramine	25–200	10–30	(NE » 5-HT > DA) A desmethyl metabolite of imipramine, effects and side effects similar to those of imipramine
Protriptyline	5–60	55–200	(NE > 5-HT > DA) Reported to improve vigilance measures anticholinergic effects
Clomipramine	10–150	15–60	(5-HT > NE » DA) Digestive problem, dry mouth, sweating, tiredness, impotence; anticholinergic effects; desmethyl-clomipramine (NE » 5-HT > DA) is an active metabolite.
SSRIs			
Fluoxetine	20–60	24–72	No anticholinergic or antihistaminergic effects, good anticatataptic effect but less potent than clomipramine; Active metabolite norfluoxetine has more adrenergic effects
Fluvoxamine	50–300	15	No active metabolite, pharmacological profile similar to fluoxetine, less active than clomipramine, gastrointestinal side effects
SNRIs			
Venlafaxine	150–375	4	New serotonergic and adrenergic uptake blocker; no anticholinergic effects, effective on cataplexy and sleepiness, nausea
Milnacipran	30–50	8	New serotonergic and adrenergic uptake blocker; no anticholinergic or antihistaminergic effects, effective on cataplexy
Atomoxetine NRI	40–60 ^a	5.2	Normally indicated for ADHD

Abbreviations: SSRI, selective serotonin reuptake blocker; NSRI, norepinephrine/serotonin reuptake inhibitor; NRI, norepinephrine reuptake inhibitor. Reboxetine is another NRI but is not available in the U.S.

^aDoses for treatments for ADHD suggested to start with smaller doses for anticatataptic treatment. GABA (GHB, sodium oxybate) is also reported to be effective on cataplexy and may act via GABA_B or via specific GHB receptors. Reduces dopamine release.

This is however rarely a significant problem with therapeutic doses. Tricyclic drugs inhibit the metabolism of amphetamine and amphetamine-like stimulants and enhance their behavioral effects [74]. However, in practice, amphetamine, 10–16 mg (and also methylphenidate, 10–60 mg, and mazindol, 2–12 mg), has been given safely with imipramine and clomipramine and at a dose range of 10–100 mg to treat narcolepsy–cataplexy [75]. The dosage of amphetamine required to control EDS in narcolepsy may be reduced by one-third through the simultaneous use of tricyclic drugs. MAO_A inhibitors (e.g., nialamide, pargyline, and tranylcypromine), inhibit the removal of amphetamine by the liver and greatly potentiate the behavioral effects of amphetamine. Consequently, coadministration of MAO inhibitors (MAOIs) and amphetamine derivatives is generally contraindicated.

3.7 NONAMPHETAMINE STIMULANTS

3.7.1 Modafinil

Modafinil (2-[(diphenylmethyl)sulfinyl]acetamide) (Fig. 3.3) is a chemically unique compound developed in France. Modafinil has been available in France since 1984 on a compassionate mode and was approved in France in 1992. Modafinil has recently been approved in the United States for the treatment of narcolepsy, idiopathic hypersomnia, shiftwork disorder, and residual sleepiness in treated patients with the sleep apnea syndrome. Modafinil is a primary metabolite of adrafinil, a vigilance-promoting compound discovered in France in 1974. The kinetic study of adrafinil led to the identification of modafinil in 1976. Modafinil lacks adrafinil's terminal amide hydroxy group (Fig. 3.3) and is better tolerated.

Modafinil is rapidly absorbed but slowly cleared. It has fairly high protein binding and a V_d of 0.8 L/kg. Its half-life is 11–14 h. Up to 60% of modafinil is converted to modafinil acid and modafinil sulfone, both of which are inactive metabolites. Metabolism primarily occurs via cytochrome P450 3A4/5, but the compound has also been reported to induce cytochrome P450 2C 19 *in vitro*. Modafinil is currently available as a racemic mixture of two active isomers. In humans, the D-isomer is cleared three times faster than the L-isomer, and females clear modafinil faster than males. The two isomers may also have slightly different pharmacodynamic properties and the longer acting isomer is currently under development.

Modafinil is one of the few compounds that have been specifically developed for the treatment of narcolepsy. Early trials include a clinical trial by Bastujji and Jouvet [76] in 1988, and the first double-blind multicenter trial in three French centers and one Canadian center in 1994 [77] have shown that 100–300 mg modafinil is effective in improving daytime sleepiness in narcolepsy and hypersomnia without interfering with nocturnal sleep but has limited efficacy on cataplexy and other symptoms of abnormal REM sleep. Modafinil is well tolerated and the most frequent reported side effects are headache, nausea, rhinitis, and nervousity (in descending order). Early clinical trials in French and Canadian pharmacological experiments in canine narcolepsy also demonstrated that modafinil has no effects on cataplexy, while it significantly increases time spent in wakefulness [78]. A recent double-blind trial in 18 centers in the United States on 283 narcoleptic subjects revealed that 200 and 400 mg of modafinil significantly reduced EDS and improved patients' overall clinical

condition. Modafinil is well tolerated and the most frequent reported side effects are headache and nausea [79].

Several factors make modafinil an attractive alternative to amphetamine-like stimulants. First, animal studies suggest that the compound does not affect blood pressure as much as amphetamines do; only high doses (800 mg) have been found to be associated with higher rates of tachycardia and hypertension. This suggests that modafinil might be useful for patients with a heart condition or high blood pressure. Second, data obtained to date suggest that tolerance and dependence is limited with this compound [76], although a recent animal study suggests cocaine-like discriminative stimulus and reinforcing effects of modafinil in rats and monkeys, respectively. Third, modafinil also has little effect on the neuroendocrine system. A comparison of healthy volunteers who were sleep deprived for 36 h versus those who received modafinil during sleep deprivation found no difference in cortisol, melatonin, or growth hormone levels. Fourth, clinical experience suggests that the alerting effects of modafinil might be qualitatively different from those observed with amphetamine [76]. In general, patients feel less irritable and/or agitated with modafinil than with amphetamines [76] and do not experience severe rebound hypersomnolence once modafinil is eliminated. This differential profile is substantiated by animal experiments. In rats and dogs, modafinil does not increase locomotion beyond the effect expected in association with increased wakefulness [78, 80]. Similarly, modafinil acutely decreases both REM and non-REM sleep in rats for up to 5–6 h, but the effect is not followed by a rebound hypersomnolence. This profile contrasts with the intense sleep recovery seen following amphetamine-induced wakefulness. Considering the many advantages of modafinil over amphetamine treatment (fewer cardiovascular side effects, lower abuse potential, tolerance, and less rebound sleepiness when the drug effects are waning), modafinil has replaced amphetamine-like stimulants as a first-line treatment for EDS.

Current indications for modafinil include narcolepsy and idiopathic hypersomnia, and it has recently been approved by the Food and Drug Administration (FDA) for the treatment of shift work disorder and residual sleepiness in treated sleep apnea patients [generally with continuous positive airway pressure (CPAP)]. There are several reports suggesting that modafinil is also effective for treatment of ADHD, fatigue in multiple sclerosis, and EDS in myotonic dystrophy or Prader–Willi syndrome. Modafinil is also being used in the treatment of recurrent hypersomnia.

The mechanism of action of modafinil is highly debated. An interaction and/or involvement of adrenergic α_1 systems was initially suggested by the ability of the α_1 antagonist prazosin to antagonize modafinil-induced increases in motor activity in mice [81] and wakefulness in cats [82]. However, modafinil does not bind to α_1 receptors *ex vivo* ($K_i > 10^{-3}$ M, obtained from prazosin binding using canine cortex) (see [78]). Furthermore, previous studies in the canine model of narcolepsy have shown that adrenergic α_1 agonists potently reduce cataplexy with a significant acute hypertensive effect. The fact that modafinil has no anticataplectic activity and lacks hypertensive effects rather suggests that its alerting properties are not derived from adrenergic α_1 stimulation.

A serotonergic 5-HT₂ receptor-mediated change in γ -aminobutyric acid (GABA)–ergic transmission was next suggested [83]. Modafinil increases 5-HT metabolism in the striatum and reduces GABA flow to the cortex [83]. The effect on GABA release is blocked by ketanserin (a 5-HT₂ antagonist) but not by prazosin [83]. Furthermore,

muscimol, a GABAergic agonist, blocks the effect of modafinil on wakefulness in cats [82]. Although a serotonergic–GABAergic interaction may be involved in the mode of action of modafinil, the effects described may be indirect and additional work is needed to substantiate this hypothesis. As for the 5-HT₂ receptor, modafinil does not bind to serotonergic receptors *in vitro*.

In the canine model of narcolepsy, it was observed that selective dopaminergic reuptake inhibitors have no effect on canine cataplexy but potently promote wakefulness (Fig. 3.5) [67]. Modafinil had a similar profile, and it was subsequently found that modafinil has a low but selective affinity for the DAT [67]. A lack of wake-promoting effects of modafinil (as well as amphetamine) in DAT KO mice clearly demonstrates that an intact DAT molecule is required for mediating modafinil's arousal effect [70].

Other investigators have shown, however, that modafinil can be distinguished pharmacologically from most other compounds with presynaptic dopaminergic activity. For example, modafinil does not produce stereotypic behavior at high doses. Additionally, agents that inhibit dopaminergic function such as D₁ blockers, D₂ blockers, and tyrosine hydroxylase blockers have no effect on modafinil's locomotor-enhancing effects in mice. Finally, an *in vitro* voltammetry study found that modafinil did not increase the catechol oxidation peak height (an indirect measure of dopaminergic activity), suggesting a lack of presynaptic dopaminergic involvement of modafinil activity. Ferraro et al. [84], however, reported that systemic administration of modafinil (30–300 mg/kg) dose dependently increased DA release in the nucleus accumbens in rats, but these authors claimed that the DA-releasing action of modafinil was most likely secondary to its ability to reduce local GABAergic transmission.

Not only is the exact molecular target of modafinil action uncertain, but also there is much debate regarding modafinil's neuroanatomical site of action. Anatomical studies coupled with functional markers of neuronal activity (i.e., the immediate early gene product Fos) have been used to determine activation patterns induced by modafinil in comparison with other stimulants [85]. In cats, amphetamine and methylphenidate induce c-Fos throughout the cortex, striatum, and other brain regions. In contrast, modafinil induces a much more restricted pattern of neuronal activation, with marked expression of c-Fos in neurons of the anterior hypothalamus area and suprachiasmatic nuclei—brain regions that have been implicated in sleep and circadian regulation [85]. Modafinil also increases c-Fos expression in hypocretin cells [35, 86] and histaminergic cells of the tuberomammillary nucleus; these effects have been suggested to mediate the wake-promoting effects of modafinil. At higher doses, the striatum and cingulate cortex are also activated [86]. Of note, however, it is likely that the stimulation of hypocretin cells is not essential to induce wakefulness since both *hcrtr2* mutated canine narcolepsy and hypocretin-ligand-deficient human narcolepsy (found in 90% of narcolepsy–cataplexy patients) respond well to modafinil treatment. More likely, activation of these cell groups is secondary to the expression of increased wakefulness, as c-Fos expression in these cell groups increases in naturally occurring wakefulness.

Gallopini et al. recently reported that modafinil inhibits the sleep-active neurons of the ventrolateral preoptic nucleus (VLPO; a sleep-promoting network of neurons) by facilitating adrenergic neurotransmission [87]. In this study, modafinil potentiated the inhibitory effects of NE on VLPO neurons in a slice preparation. Surprisingly,

modafinil did not potentiate the inhibitory effects of DA or 5-HT on VLPO neurons. Nisoxetine, a potent NET inhibitor with low affinity to DAT [67], had a similar effect and the response to the two drugs was not addictive, suggesting they might work through the same biochemical pathways. Since modafinil does not bind to the NET [67] and NE uptake inhibitors do not possess strong wake-promoting effects, modafinil may modulate NE/DA uptake mechanisms through novel mechanisms. In this case, modafinil may work on both the DA and NE system to promote wakefulness, and adrenergic–DAT interactions may be involved. Of note, however, very high modafinil concentrations (generally 200 μ M, the maximum that can be dissolved) were used in this study *in vitro*, and it may also be that at this very high dose small effects on adrenergic uptake, undetectable with the usual radio receptor binding assays, could occur.

Further studies of the action of modafinil will be needed to clarify the above discrepancies regarding its modes of action and may lead to new and interesting insights into the mode of action of stimulant medications in general.

3.7.2 Mazindol

Mazindol (2–8 mg daily), a sympathomimetic anorectic agent, is rarely used in the United States. At these doses, mazindol produces central stimulation, a reduction in appetite, and an increase in alertness but has little or no effect on mood or the cardiovascular system. Mazindol is a weak releasing agent for DA that also blocks both DA and NE reuptake. Mazindol has a high affinity for the DA and NE transporters (see [67]), yet interestingly this compound has a low abuse potential. It is effective for the treatment of both EDS and cataplexy in humans [88] and in canine narcolepsy [89], possibly due to its dual dopaminergic and noradrenergic effects [67]. Narcoleptics are relatively well tolerated to mazindol, and reported side effects are insomnia, headache, anorexia, constipation, chest discomfort, and delayed menstruation, but drug tolerance may occur in some patients. However, higher doses of mazindol are reported to cause other side effects, including gastrointestinal discomfort, nervousness, dry mouth, nausea, urinary retention, vomiting, tremor, and angioneurotic edema. Therefore, mazindol may not be recommended for treatment of severe narcoleptic subjects that require large doses.

3.7.3 Bupropion

Bupropion is a DA reuptake inhibitor that may be useful for the treatment of EDS associated with narcolepsy (100 mg t.i.d.) [67, 90]. It may be especially useful in cases associated with atypical depression [90]. Bupropion was synthesized in 1966 by a group seeking new antidepressants chemically related to tricyclic antidepressants but without any significant sympathomimetic, cholinolytic, or MAOI properties. Bupropion is classified as a monocyclic phenylbutylamine of the aminoketone group. It selectively blocks DA uptake and is 6 times more potent than imipramine and 19 times more potent than amitriptyline in blocking DA reuptake. The selectivity of bupropion for the DAT is not absolute. Bupropion is a weak competitive inhibitor of NE reuptake (65-fold less potent than imipramine), and very limited serotonergic effects are also observed (200-fold less potent than imipramine). Bupropion is generally well tolerated, and the most commonly reported side effects include

headache, nausea, dry mouth, and insomnia. Convulsion is a dose-dependent risk of bupropion (0.1% at 100–300 mg and 0.4% at 400 mg).

3.7.4 Selegiline (L-Deprenyl)

Selegiline is a methamphetamine derivative and a potent, irreversible MAO_B selective inhibitor primarily used for the treatment of Parkinson's disease. Low doses of selegiline do not necessitate dietary restriction as usually required with other irreversible MAOIs. Ten milligrams of selegiline daily has no effect on the symptoms of narcolepsy, but 20–30 mg improves alertness and mood and reduces cataplexy, an effect comparable to D-amphetamine at the same dose [91]. Selegiline may be an interesting alternative to the use of more classical stimulants, as its potential for abuse has been reported to be very low.

The fact that this compound is often considered as a simple MAO_B inhibitor rather than an amphetamine precursor deserves special mention. Selegiline does not only inhibit MAO_B irreversibly, it also metabolizes into amphetamine (20–60% in urine) and methamphetamine (9–30% in urine). In the canine model of narcolepsy, selegiline (2 mg/kg p.o.) was demonstrated to be an effective anticataplectic agent, but this effect was found to be mediated by its amphetamine metabolites rather than via MAO_B inhibition [92]. Several trials in human narcolepsy have demonstrated a good therapeutic efficacy of selegiline on both sleepiness and cataplexy with relatively few side effects [91, 93], but this efficacy is also likely to be partially mediated by amphetamine metabolites.

3.7.5 Caffeine

Caffeine may be the most popular and widely consumed CNS stimulant in the world. An average cup of coffee contains 50–150 mg of caffeine. Tea, cola drinks, chocolate, and cocoa all contain significant amounts of caffeine. Caffeine can also be bought over the counter (OTC) (Nodoz, 100 mg caffeine; Vivarin, 200 mg caffeine) and is commonly used by narcoleptic patients prior to diagnosis.

Taken orally, caffeine is absorbed rapidly. The half-life of caffeine is 3.5–5 h. The behavioral effects of caffeine include increased mental alertness, a faster and clearer flow of thought, wakefulness, and restlessness. Fatigue is reduced and sleep onset delayed [94]. The physical effects of caffeine include palpitations, hypertension, increased gastric acid secretion, and increased urine output [94]. Heavy consumption (12 or more cups a day, or 1.5 g of caffeine) causes agitation, anxiety, tremors, rapid breathing, and insomnia.

Caffeine is a xanthine derivative. The mechanism of action of caffeine on wakefulness involves nonspecific adenosine receptor antagonism. Adenosine is an endogenous sleep-promoting substance with neuronal inhibitory effects. In animals, sleep can be induced after administration of metabolically stable adenosine analogs with adenosine A₁ receptors (A₁R) or A_{2A} receptors (A_{2A}R) agonistic properties, such as *N*6-L-(phenylisopropyl)-adenosine, adenosine-5'-*N*-ethylcarboxamide, and cyclohexyladenosine [95]. Adenosine content is increased in the basal forebrain after sleep deprivation. Adenosine has thus been proposed to be a sleep-inducing substance accumulating in the brain during prolonged wakefulness [96].

Most studies in the area of sleep and adenosinergic effects have focused on A1R-mediated effects. The rationale for this focus is that A1R is widely distributed in the CNS, whereas A2AR is discretely localized in the striatum, nucleus accumbens, and olfactory bulb. Interestingly, sleep–wake patterns and response to sleep deprivation were recently examined in A1R KO mice and found to be generally unaltered, suggesting that the constitutional lack of A1R does not prevent homeostatic regulation of sleep. In contrast, the sleep inhibitory effects of 8-cyclopentyltheophylline (a selective A1R antagonist) were abolished in these animals, indicating A1R mediation of stimulant effects with this compound.

3.8 FUTURE STIMULANT TREATMENTS

It is now revealed that human narcolepsy is caused by a dramatic decrease in hypocretin levels in the brain and CSF. This raises the possibility that hypocretin-based stimulant therapies may be designed in the future. Recent experiments in canine narcolepsy (receptor mutated and ligand deficient) suggest that stable and centrally active hypocretin analogs (possibly nonpeptide synthetic hypocretin ligands) will need to be developed in order to be effective peripherally [97]. This is also substantiated by a recent study that found normalization of sleep/wake patterns and behavioral arrest episodes (equivalent to cataplexy and REM sleep onset) in orexin/hypocretin—deficient mice models supplemented by central administration of hypocretin-1 [98] (see Section 3.12).

Another potential drug development in the area of wake promotion is with histaminergic compounds. Histamine has long been implicated in the control of vigilance, and H₁ antagonists are strongly sedative. The downstream effects of hypocretins on the histaminergic system (*Hcrtr2* excitatory effects) are likely to be important in mediating the wake-promoting properties of hypocretin [49]. In fact, brain histamine and CSF histamine contents are reduced in narcoleptic subjects [52, 99]. Although centrally injected histamine or histaminergic H₁ agonists promote wakefulness, systemic administrations of these compounds induce various unacceptable side effects via peripheral H₁ receptor stimulation. In contrast, the histaminergic H₃ receptors are regarded as inhibitory autoreceptors and are enriched in the CNS. H₃ antagonists enhance wakefulness in normal rats and cats [100] and in narcoleptic mice models [101]. Histaminergic H₃ antagonists might be useful as wake-promoting compounds for the treatment of EDS or as cognitive enhancers and are under study in several pharmaceutical companies.

Another possible area with less current pharmaceutical interest is the use of thyrotropin-releasing hormone (TRH) direct or indirect agonists. TRH itself is a small peptide which penetrates the blood–brain barrier at very high doses. Small molecules with agonistic properties and increased blood–brain barrier penetration (i.e., CG3703, CG3509, or TA0910) have been developed, partially due to the small nature of the starting peptide [102]. TRH (at the high dose of several milligrams per kilogram) and TRH agonists increase alertness and have been shown to be wake promoting and antiepileptic in the narcoleptic canine model [103, 104], and it has excitatory effects on motoneurons [105]. Initial studies had demonstrated that TRH enhances DA and NE neurotransmission [106, 107], and these properties may partially contribute to the wake-promoting and antiepileptic effects of TRH.

Interestingly, recent studies have suggested that TRH may promote wakefulness by directly interacting with the thalamocortical network: TRH itself and TRH receptor type 2 are abundant in the reticular thalamic nucleus [108]. The local application of TRH in the thalamus abolishes spindle wave activity [109], and in the slice preparations, TRH depolarized thalamocortical and reticular/perigeniculate neurons by inhibition of leak K^+ conductance [109].

Other pathways with possible applications in the development of novel stimulant medications include the adenosinergic system (see above), the adrenergic system (e.g., some NE reuptake inhibitors), the GABAergic system (e.g., inverse benzodiazepine agonists), and the glutamatergic system (ampakines) (see [7]).

3.9 PHARMACOLOGICAL TREATMENT OF CATAPLEXY

3.9.1 Historical Overview of Antidepressants

Since the 1960s, it has been known that imipramine is very effective for reducing cataplexy [110]. Together with protriptyline and clomipramine, these tricyclic antidepressants are still the most commonly used anticataplectic agents (Table 3.3).

Antidepressants are drugs that are effective for treating depression and are represented by a diverse group of chemical structures. Many hypotheses about how these antidepressants work exist, and usually these theories involve biogenic amine neurotransmitters, especially NE and 5-HT [111].

Of the many antidepressants, some of the first types discovered and, up until about 10 years ago, the most widely prescribed were the tricyclics, so named because of the three fused rings in the structure. The prototype of this class of compound, imipramine, was initially synthesized for use as an antihistamine (H_1) but was discovered to have antidepressant properties as the result of astute clinical observations on depressed schizophrenic patients [111]. Imipramine was selected for a trial in schizophrenia because of its close structural similarity to chlorpromazine. This finding led to the study of imipramine in clinical trials in depressed patients which subsequently proved its efficacy as an antidepressant [111].

Another class of antidepressants, the MAOIs, was introduced in the 1950s because of observations in both the clinic and laboratory [112]. The first compound of this group to be tested and proven effective as an antidepressant was iproniazid, an antituberculosis drug. This research began after scientists observed that iproniazid caused euphoria and elation in some patients treated for tuberculosis and that it reversed the apparent sedation caused by the drug reserpine in laboratory animals. MAOIs are used much less frequently than other types of antidepressants because of the “cheese reaction,” a marked and potentially fatal increase in blood pressure that can occur when a patient being treated with one of these drugs ingests foods with high tyramine content (such as aged cheese, bananas, smoked and pickled fish, fermented sausages, and red wine, to list a few) [112]. The excess tyramine, which cannot be degraded by MAO, displaces neuronal stores of catecholamines (e.g., NE) that ultimately increase blood pressure. Consequently, patients taking MAOIs are required to be on a tyramine-free diet and must also avoid the use of sympathomimetic stimulants.

Shortly after the discovery of tricyclic antidepressants, it was shown that these drugs acutely blocked reuptake of both NE and 5-HT by the nerve ending [111]. In general, tertiary amine tricyclic antidepressants, such as imipramine or clomipramine, were potent blockers of 5-HT reuptake but weak blockers of NE transport, whereas the converse seemed true for the secondary amine compounds [113]. It was also shown that MAOIs increase brain levels of catecholamines and 5-HT by preventing their degradation [112]. Thus, it was thought that the therapeutic effect of the antidepressant was due to increased duration of elevated levels of monoamines in the synaptic cleft (i.e., the monoamine hypothesis), but the exact mechanisms of the action of antidepressants on depressive symptoms are not known [111]. One of the complex issues is the fact that the mood-elevating effect of antidepressants does not become apparent until at least 7–10 days to several weeks after treatment has started; longer term mechanisms need to be considered. This sharply contrasts to an immediate occurrence of antiepileptic effects by these compounds when they are used in narcoleptic patients [7].

Most classical tricyclics also have the ability to block several neurotransmitter receptors, such as at histaminergic H_1 , muscarinic acetylcholinergic, α_1 -adrenergic, and serotonergic 5-HT_{2A} receptors [111]. These pharmacological effects occur immediately and are probably not related to its major mode of action but underlie various adverse effects. For example, muscarinic receptor blockade can cause dry mouth and constipation, and histamine H_1 receptor antagonism can cause sedation and drowsiness. Some antidepressants (such as the tricyclic antidepressant doxepin) have such high affinity for the H_1 receptor that they are among the most potent histamine H_1 antagonists available. Thus, doxepin is also used for the treatment of allergy and certain dermatological problems.

3.9.2 Tricyclic (First-Generation) Antidepressants

First-generation tricyclic antidepressants, such as imipramine, protriptyline, and clomipramine, potentially reduce cataplexy in many subjects and therefore are still the most commonly used antiepileptic agents (Table 3.3). Similar to the situation seen in patients with depression, the use of tricyclic antidepressants in the treatment of cataplexy is, however, also hampered by a number of problems. The first is the relatively poor side-effect profile of most tricyclic compounds. These are mostly due to their anticholinergic properties, thus leading to dry mouth (and associated dental problems), tachycardia, urinary retention, constipation, and blurred vision (see Table 3.3). This profile was initially thought to be involved in antiepileptic effects of these tricyclics. However, animal experiments as well as findings with newer antidepressants in human narcolepsy suggest that this property is not required for the antiepileptic property of antidepressants. Additional side effects are weight gain, sexual dysfunction (impotence and/or delayed orgasm), tremors, antihistamine effects leading to sedation, and occasionally orthostatic hypotension due to the α_1 -adrenergic blockade of some compounds. Nighttime sleep might also become more disturbed due to increased muscle tone and leg movements [60].

As described earlier, the cardinal pharmacological property of tricyclic antidepressants is their ability to inhibit the reuptake of NE (and epinephrine) and 5-HT [111]. The degree of reuptake inhibition of NE and 5-HT is quite variable depending on the compound and on the existence of active metabolites (mostly active on

adrenergic reuptake). Active metabolites often have longer half-lives and tend to accumulate during chronic drug administration. The metabolic half-lives of these compounds also vary among individuals. Additionally, some tricyclic compounds (such as protriptyline), are also weak DA reuptake inhibitors [111]. Thus, it is not simple to conclude which pharmacological property is important for the anticataplectic effects in humans.

3.9.3 Second- to Third-Generation Antidepressants

The newer second-generation antidepressants usually are more selective at blocking the reuptake of 5-HT over NE. This has led to the concept of the serotonin-selective reuptake inhibitor (SSRI) [114]. The first of this group and the most widely prescribed until recently was fluoxetine, which was first marketed in the United States in 1988. The introduction of newer antidepressants with selective serotonergic reuptake inhibition properties and no anticholinergic effects (i.e., SSRIs), (such as fluoxetine, fluvoxamine, paroxetine, sertraline, femoxamine, zimelidine, and citalopram) has raised hope that the control of cataplexy can be achieved with fewer side effects. In general, however, clinicians have been less impressed with the anticataplectic effects of the SSRIs than those of classical tricyclics [115, 116]. Among these compounds, fluoxetine at the 20- to 60-mg dose is a viable alternative to tricyclic compounds [115]. Fluoxetine has a good side-effect profile and induces anorexia rather than weight gain, a significant advantage for some patients.

Another compound, venlafaxine, is marketed as a 5-HT and NE reuptake inhibitor (SNRI) antidepressant, and it reduced cataplexy and sleepiness in a small pilot study. Another SNRI, milnacipran, is available in some European countries and Japan, and this compound reduces cataplexy in humans and canines (experiments in progress). A selective NE blocker, atomoxetine (developed under the name of tomoxetine), was also introduced in the U.S. market and used for the treatment of ADHD. Yet another selective NE blocker, reboxetine, is only available in some European countries. Although no published data are yet available, it may be interesting to evaluate the anticataplectic effects of selective NE blockers such as atomoxetine and reboxetine.

Because the newer second-generation drugs usually have lower affinities for the above-mentioned receptors, they are also less likely to cause the adverse effects commonly seen with the older antidepressants [114].

3.9.4 Mechanisms of Action of Tricyclic Anticataplectics

Neuropharmacological understandings of cataplexy also have been greatly facilitated using these canine models [7]. Since cataplexy can be easily elicited by food or play and the severity of cataplexy can be quantified by a simple behavioral assay (i.e., food-elicited cataplexy test), these animals have been intensively used for evaluating anticataplectic effects of various compounds [7].

Although the mechanism for the induction of cataplexy is not identical to that for REM sleep, both likely share common physiological and pharmacological mechanisms, especially for the executive systems for muscle atonia. Thus, the understanding of the pharmacological control of REM sleep is essential to the understanding of cataplexy.

The importance of increased cholinergic activity in triggering REM sleep or REM sleep atonia is well established (see [117]). Similarly, activation of the cholinergic systems using the acetylcholinesterase inhibitor physostigmine also greatly exacerbates cataplexy in canine narcolepsy, but with various side effects [7]. This cholinergic effect is mediated via muscarinic receptors since muscarinic stimulation aggravates cataplexy, while its blockade suppresses it, and nicotinic stimulation or blockade has no effect [7]. Application of muscarinic antagonists in human narcolepsy is, however, hampered due to its peripheral side effects.

Monoaminergic transmission is also critical for the control of cataplexy. All therapeutic agents currently used to treat cataplexy (i.e., antidepressants or MAOIs) are known to act on these systems. Furthermore, whereas a subset of cholinergic neurons are activated during REM sleep, the firing rate of monoaminergic neurons in the brain stem (such as in the LC and the raphe nucleus) is well known to be dramatically depressed during this sleep stage [118]. In contrast, dopaminergic neurons in the VTA and SN do not significantly change their activity during natural sleep cycles [118]. Using canine narcolepsy, it was recently demonstrated that adrenergic LC activity is also reduced during cataplexy [119].

3.9.5 Preferential Involvement of Adrenergic Neurotransmission in Control of Canine Cataplexy

As mentioned above, tricyclic antidepressants have a complex pharmacological profile that includes monoamine reuptake inhibition, anticholinergic, α_1 -adrenergic antagonistic, and antihistaminergic effects, making it difficult to conclude which of these pharmacological properties is actually involved in their anticataplectic effects.

In order to determine which property was most relevant, we studied the effects of a large number (a total of 17 compounds) of reuptake blockers/release enhancers specific for the adrenergic, serotonergic, or dopaminergic systems. Adrenergic reuptake inhibition was found to be the key property involved in the anticataplectic effect [120]. Serotonergic reuptake blockers were only marginally effective at high doses and the dopaminergic reuptake blockers were completely ineffective. Interestingly, it was later found that these DA reuptake inhibitors had potent alerting effects in canine narcolepsy [7] (see also Section 3.6.1.2).

We also compared the effects of several antidepressants with those of their demethylated metabolites. Many antidepressants (most typically tertiary amine tricyclics) are known to be hepatically first-pass metabolized into their demethylated metabolites that have longer half-lives and higher affinities for adrenergic reuptake sites [121]. During chronic drug administration, these demethylated metabolites accumulate [121] and can thus be involved in the drug's therapeutic action. The effects of five available antidepressants (amitriptyline, imipramine, clomipramine, zimelidine, and fluoxetine) were compared with those of their respective demethylated metabolites (nortriptyline, desipramine, desmethylclomipramine, norzimelidine and norfluoxetine) [113]. In all cases, the demethylated metabolites were found to be more active on cataplexy than were the parent compounds. We also found that the active dose of all anticataplectic compounds tested positively correlated with the *in vitro* potency of each compound to the adrenergic transporter but not with that of the serotonergic transporter [113]. In fact, the anticataplectic effects were negatively correlated with the *in vitro* potency for serotonergic reuptake inhibition, but this may

be biased since potent adrenergic reuptake inhibitors included in the study have a relatively low affinity to serotonergic reuptake sites. Although most of these results were obtained from inbred *hcrtr2*-mutated narcoleptic Dobermans, similar findings (the preferential involvement of adrenergic system) have also been obtained in more diverse cases of sporadic canine narcolepsy in various breeds donated to our colony (see [122]).

The fact that serotonergic reuptake blockers, also known to have inhibitory effects on REM sleep, have less or no effect on cataplexy is surprising. Like adrenergic cells of the LC, serotonergic cells of the raphe nuclei dramatically decrease their activity during REM sleep [117]. This discrepancy could be explained by a preferential effect of serotonergic projections on REM sleep features other than atonia, for example, in the control of eye movements. In this model, adrenergic projections may be more important than serotonergic transmission in the regulation of REM sleep atonia and thus cataplexy [120]. In favor of this hypothesis, a recent experiment has shown that serotonergic activity does not decrease during cataplexy in narcoleptic canines [123].

3.9.6 Receptor Subtypes Involved in Control of Cataplexy

In order to dissect receptor subtypes that significantly modify cataplexy, more than 200 compounds with various pharmacological properties (cholinergic, adrenergic, dopaminergic, serotonergic, prostaglandins, opioids, benzodiazepines, GABAergics, and adenosinergics) have also been studied in the narcoleptic canine model (see [7] for details). Although many compounds (such as M_2 antagonists, α_1 agonists, α_2 antagonists, dopaminergic D_2/D_3 antagonists, 5-HT_{1A} agonists, TRH analogs, prostaglandin E₂, and L-type Ca^{2+} channel blockers) reduce cataplexy, very few compounds significantly aggravate cataplexy (cataplexy-aggravating effects are assumed to be more specific, since cataplexy can be nonspecifically reduced by unpleasant drug sideeffects) [7]. Among the monoaminergic receptors, blockade of the postsynaptic adrenergic α_{1B} receptors [124] and stimulation of presynaptic α_2 autoreceptors [125] were also found to aggravate cataplexy, a result consistent with a primary adrenergic control of cataplexy. We also found that small doses of DA D_2/D_3 agonists significantly aggravated cataplexy and induced significant sleepiness in these animals [126, 127]. The cataplexy-inducing effects of D_2/D_3 agonists are, however, difficult to reconcile considering the fact that dopaminergic reuptake blockers (in contrast to adrenergic reuptake inhibitors) have absolutely no effect on cataplexy [120] (see also Section 3.9.5). We also found recently that sulpiride (a D_2/D_3 antagonist) significantly suppresses cataplexy in the canine model but has no effect on REM sleep [128]. D_2/D_3 agonists are clinically used for the treatment of human periodic leg movements during sleep (PLMS). Incidence of PLMS is high in human narcolepsy, and it also occurs in the narcoleptic Doberman [129]. The dopaminergic system (i.e., D_2/D_3 receptor mechanisms) may thus be specifically involved in sleep-related motor control rather than REM sleep.

The sites of action of D_2/D_3 agonists were also investigated by local drug perfusion experiments, and a series of experiments identified acting sites for these compounds. These include dopaminergic nuclei or cell groups, such as the VTA [127], SN [73], and A11 [130] (a diencephalic DA cell group that directly projects to the spinal ventral horn), suggesting a direct involvement of DA cell groups and DA cell body autoreceptors in the regulation of cataplexy.

The mechanism for emotional triggering for cataplexy remains to be studied, but it is possible that multiple brain sites and multiple functional and anatomical systems are involved.

3.9.7 Monoamine Oxidase Inhibitors

MAOIs are known to potently reduce REM sleep and are therefore candidate anticataplectic agents. This has led several investigators to use MAOIs for the treatment of narcolepsy [131, 132]. The extracellular effect of naturally released catecholamine is terminated by either reuptake or enzymatic degradation, either by MAO and/or catechol-*O*-methyl transferase. MAO is a flavin-containing dominating enzyme located in the outer membranes of neural and glial mitochondria (see [112]). It exists as two forms: MAO_A, blocked by clorgyline and with high affinity for noradrenaline and 5-HT, and MAO_B, insensitive to clorgyline and with high affinity for phenylethylamine and DA (see [112]).

Even if these compounds are clearly active on narcolepsy [131, 132] and may be useful in cases refractory to more conventional treatment, the first-generation MAOIs are rarely used in clinical practice to date due to their poor safety profile (e.g., the cheese effect) (see [132a]). It is also dangerous to use other drugs with sympathomimetic effects (tricyclic antidepressants, amphetamine-like compounds, or simply catecholaminergic cardiac stimulants) in patients treated with MAOIs due to the existence of interactions (sometimes fatal) that are impossible to predict (see [112]). Other side effects include edema, impotence, weight gain, insomnia, long-term hypertension, and psychological disturbances (see [112]). Drug withdrawal may lead to REM sleep rebound with exacerbation of the narcolepsy and the development of vivid nightmares [131]. In addition, the first generation of MAOI (irreversible MAOIs) had the unique property of binding covalently to the active site of the enzyme ("suicide substrate"), thus leading to long-term (up to several weeks) enzymatic inhibition even after a single dose (see [112]).

A safer generation of MAOIs is now becoming available. These include compounds with selective MAO_A or MAO_B inhibition and/or a reversible enzymatic inhibition profile. In contrast to irreversible MAOIs, reversible MAOIs are substrates for the MAOs and compete with the endogenous monoamines (see [112]). Some of these new reversible MAOIs (brofaromine, moclobemide) are now being used in clinical trials in Europe and seem to be effective and safe for the treatment of human narcolepsy without noticeable side effects [133]. These compounds can be used with minimal dietary precautions.

Selegiline is a potent irreversible MAO_B selective inhibitor used in the treatment of Parkinson's disease. This compound is essentially a methamphetamine derivative and is metabolized to a significant extent into amphetamine and methamphetamine (9–30 and 20–60% are found in urine, respectively); the use of low doses of selegiline does not require dietary restriction. Ten milligrams of selegiline daily has no effect on the symptoms of narcolepsy, but 20–30 mg improves alertness and mood and reduces cataplexy somewhat, an effect comparable to amphetamine at the same dose [91]. Indeed, an experiment in canines also suggests that the anticataplectic effects of selegiline are likely to be explained by its active metabolites, *L*-amphetamine and *L*-methylanphetamine [92].

3.9.8 Sodium Oxybate

Sodium oxybate (GHB), taken in the evening and once again during the night reduces cataplectic attacks and other manifestations of REM sleep [134, 135]. Its elimination half-life is 1–2 h, and it increases NREM sleep stages 3 and 4, decreases nighttime awakenings, and consolidates fragmented REM sleep [135, 136]. Recent studies have demonstrated a measurable improvement in patients' reported daytime sleepiness and a moderate reduction in cataplexy. Although improvement in sleepiness occurs relatively quickly, anticataplectic effects appeared one to two weeks after the initiation of the treatment. GHB is reported to possess inhibitory effects through GABA_B receptors, but the precise mechanisms of action of GHB on sleepiness and cataplexy are unknown. GHB given at bedtime and with a second dosage upon awakening during the night (at least 3 h before rising time) may also help consolidate nocturnal sleep. If the patient wakes up during the night, he (or she) may experience dizziness and confused states. One problem with GHB is the nonmedical use of this compound to elicit an altered state of consciousness with important social and legal implications. Its clinical use has recently been approved in the United States by the FDA.

3.10 TREATMENT OF SLEEP PARALYSIS AND HYPNAGOGIC HALLUCINATIONS

The treatment of these two symptoms is much less well codified. Hypnagogic hallucinations can be quite bothersome and often occur in patients who also suffer from frequent nightmares. As they are a manifestation of sleep onset REM sleep, the compounds that suppress REM sleep are usually helpful in alleviating this symptom, and tricyclic antidepressant treatment has been reported to have some beneficial effects [137]. Sleep paralysis only rarely requires treatment, but tricyclic antidepressants are also very effective for preventing this symptom. Recently, high doses (60 mg daily) of fluoxetine have been advocated as a very active treatment for isolated sleep paralysis [138]. GHB is also effective in suppressing both hypnagogic hallucinations and sleep paralysis [135].

3.11 TREATMENT OF DISTURBED NOCTURNAL SLEEP

Insomnia is a major complaint in narcoleptic subjects. Several studies reported that benzodiazepine hypnotics are effective in consolidating nighttime sleep in narcoleptic patients [139]. GHB (a compound with REM- and SWS- inducing properties) has also been used for consolidating nighttime sleep, an effect that leads to decreased EDS and cataplexy the following day [135]. Alternatively, this short half-life sedative hypnotic may induce rebound insomnia-like status the following day and produce alertness during daytime. Due to its positive libido effects, its SWS-enhancing properties, and the reported connection between SWS and growth hormone release, the drug is, unfortunately, widely abused by athletes and other populations [140]. The compound has also been reported to increase periodic leg movements in narcoleptic patients [141].

3.12 FUTURE ANTICATAPLECTICS

Since the occurrence of cataplexy in humans is tightly associated with hypocretin ligand deficiency, hypocretin replacement may be a new attractive treatment choice in human narcolepsy.

The effects of hypocretin-1 (hypocretin-2 has much lower in vivo biological activity, possibly due to its biological instability) administration on sleep and narcolepsy symptoms have been evaluated [97, 142]. Central administration of hypocretin-1, for example in the ventricle of wild-type rodents or normal canines, is strongly wake promoting [97]. This effect is likely to be, at least in part, mediated by the *hcrtr2* receptor, as intracerebroventricular hypocretin-1 at the same dose (10–30 nmol) did not promote wakefulness in *hcrtr2*-mutated narcoleptic canines [97]. A lack of wake-promoting effects of hypocretin-1 was also observed in *hcrtr2* KO mice (Mieda et al., personal communication).

Experiments conducted after intravenous administration of hypocretin-1 have been performed in *hcrtr2*-mutated canines and in two hypocretin-ligand-deficient narcoleptic dogs. In spite of a previous report [142], we were unable to detect any significant effect even at very high doses of hypocretin-1 in *hcrtr2*-mutated animals [97]. This result was not surprising considering the lack of effects after central administration of the same dose in these animals lacking *hcrtr2*. More interestingly, a possible very slight and short lasting suppression of cataplexy was observed in a single hypocretin-deficient narcoleptic animal at extremely high intravenous doses [97, 143]. Intrathecal administration of hypocretin-1 by implanting a Medtronic pump with catheterization of the cisterna magna was also carried out in a single hypocretin-deficient narcoleptic canine [143]. However, no significant effect on cataplexy was observed [143], probably because the hypocretin-1 did not diffuse into upper ventricular compartments. Additional studies using intraventricular rather than intracisternal injections will be needed to verify that hypocretin-deficient narcoleptic canines are responsive to supplementation.

Using hypocretin neuron-ablated narcoleptic mice (orexin/ataxin 3 transgenic mice), Mieda et al. recently demonstrated that replacement of central hypocretin by either pharmacological (intracerebroventricular injection of hypocretin-1) or genetic (ectopic expression of hypocretin in the brain) manipulations allowed for the rescue of the narcolepsy–cataplexy phenotype [98]. These results indicate that hypocretin neuron-ablated mice retain the ability to respond to hypocretin neuropeptides and that hypocretin receptors, intracellular signaling, postsynaptic neural networks, and other downstream neurotransmitter pathways remain anatomically and functionally intact. Most importantly, a temporally regulated and spatially targeted secretion of hypocretin is not likely to be necessary to prevent narcoleptic symptoms [98]. The potential treatment of narcolepsy–cataplexy symptoms in humans by using agonists for hypocretin receptors is a promising new field. Development of small-molecular-weight nonpeptide agonists will be essential for this purpose.

Experiments in canine narcolepsy have suggested that dopamine D₂/D₃ receptor mechanisms may be more specifically involved in regulation of cataplexy (and sleep-related motor control) than in REM sleep, and it is interesting to evaluate anticataplectic effects of D₂/D₃ antagonists in humans.

Several new classes of wake-promoting compounds possibly applied to human narcolepsy were discussed earlier. It is interesting to note that some of these

compounds, such as H_3 antagonists and TRH, also potently reduce cataplexy in the canine model [103, 104]. The modes of action of anticataplectic effects of these compounds are not yet known, and further evaluations of anticataplectic effects of these compounds are warranted.

3.13 CONCLUSIONS

Over 90% of diagnosed narcoleptic patients are reported to take medications to control symptoms [63]. Amphetamine-like stimulants have been used in the treatment of EDS in narcolepsy and various other conditions for decades, yet only recently has the mode of action of these drugs on vigilance been characterized. In almost all cases, the effects on vigilance were found to be mediated via the effects on the DAT. This has generally led to the widely accepted hypothesis that wake-promoting effects will be impossible to differentiate from abuse potential effects for these compounds. Importantly, however, the various medications available have differential effects and potency on the DAT and on monoamine storage/release. It thus appears more likely that complex properties [e.g., the ability to release DA rather than simply block reuptake plus the combined effects on other monoamines (such as 5-HT)] may be important to explain the characteristic of the wake-promoting effect of each compound.

The mode of action of modafinil remains controversial and may involve dopaminergic and/or nondopaminergic effects. Whatever its mode of action, the compound is generally found to be safer and to have a lower abuse potential than amphetamine stimulants. Its favorable side-effect profile has led to an increasing use outside the narcolepsy indication, most recently in the context of shift work disorder and residual sleepiness in treated sleep apnea patients. This recent success exemplifies the need for developing novel and nonamphetamine wake-promoting compounds. A need for treating daytime sleepiness extends well beyond the relatively rare indication of narcolepsy-cataplexy.

Cataplexy is currently treated with antidepressants, a class of compounds that enhance monoaminergic neurotransmission by inhibition of monoamine reuptake (NE, 5-HT, DA). Most narcolepsy-cataplexy patients also take wake-promoting compounds, but these have little effect on cataplexy. Experiments in canine narcolepsy suggest a preferential involvement of NE rather than 5-HT reuptake inhibition in the anticataplectic properties of the drugs. DA reuptake inhibition does not reduce cataplexy but significantly enhances wakefulness. In humans, compounds with NE reuptake inhibition also reduce cataplexy. SSRIs are also very commonly used as anticataplectics in the human. This is mostly due to their better side-effect profiles, but the anticataplectic effects of these compounds are rather modest. Recently, selective NE and NE/5-HT reuptake inhibitors were introduced, and evaluations of these are in progress and may bring profound beneficial effects.

MAOIs are another choice of anticataplectic medication, but use of classical, nonselective, irreversible MAOIs is hampered by various side effects, including hypertensive crisis. Reversible and selective MAOIs are available in some countries, and this may be useful for some severe or refractory cataplexy cases.

Other classes of compounds with possible applications in the development of novel stimulant/anticataplectic medications include the histamine system (especially

H₃ receptor antagonists), TRH system (TRH analogs), adenosinergic system, adrenergic system (e.g., some NE reuptake inhibitors), GABAergic system (e.g., inverse benzodiazepine agonists), and glutamatergic system (ampakines).

Finally, hypocretin replacement therapy may be more straightforward and efficient for the treatment of both cataplexy and sleepiness, but the development of small-molecular-weight peptide agonists is essential. If these are effective in humans, cell transplantation and/or gene therapy may also be developed in the near future.

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4

HYPOCRETIN/OREXIN SYSTEM

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4.1	Lateral Hypothalamus	125
4.2	Discovery of Hypocretins	126
4.3	Two Hypocretin Receptors	128
4.3.1	Agonists and Antagonists	128
4.4	Hypocretin Cell Bodies	128
4.4.1	Hcrt Projections	129
4.4.2	Hcrt at Synapses	130
4.4.3	Hcrt Afferents	130
4.4.4	Receptor Distributions	130
4.5	Hypocretins: Excitatory Neurotransmitters	131
4.6	Feeding and Metabolism	132
4.7	Autonomic and Endocrine Effects	134
4.8	Motivation and Addiction	135
4.9	Pain and Anaesthesia	136
4.10	Hippocampal Plasticity	136
4.11	Narcolepsy: Disease of Hypocretin System	136
4.12	Hypocretin and Arousal Circuitry	139
4.12.1	LH Neurons	140
4.12.2	Noradrenergic Systems	141
4.12.3	Serotonergic Systems	142
4.12.4	Histaminergic Systems	142
4.12.5	Dopaminergic Systems	143
4.12.6	Cholinergic Systems	143
4.13	Hypocretins Integrate Arousal, Feeding Behavior, and Motivation	144
	Acknowledgments	145
	References	145

4.1 LATERAL HYPOTHALAMUS

Observations on humans and experimental animals with localized hypothalamic lesions led to the earliest notions about the role of the lateral hypothalamus (LH).

Studying patients with encephalitis lethargica, von Economo [1] proposed that the posterior hypothalamus (including the LH) was required for maintaining the awake state. Ablations of the monkey LH led to coma and hypophagia, whereas ablations of the medial hypothalamus led to hyperphagia [2–5]. Thus, the LH was known to play a role in both arousal and energy balance, both important aspects of motivated behavior. Additionally, animals lever press for electrical stimulation when electrodes are placed within the LH [6], further implicating it as a reward-mediating structure. The signaling molecules and circuitry responsible for coordinating these behaviors remained unknown until the discoveries of the hypocretin (Hcr) and melanin-concentrating hormone (MCH) systems.

4.2 DISCOVERY OF HYPOCRETINS

An open-system search for undiscovered hypothalamic regulatory peptides provided the first glimpse of the hypocretins. Gautvik and colleagues [7] conducted a systematic subtractive hybridization survey aimed at identifying messenger RNA (mRNA) species whose expression was restricted to discrete nuclei within the rat hypothalamus. Among these was a species whose expression, as detected by *in situ* hybridization analyses (Fig. 4.1), was restricted to a few thousand neurons that were bilaterally distributed within the dorsolateral hypothalamus [7, 8]. The mRNA migrated in northern blots at ~700 nucleotides, detectable during brain development at low concentrations as early as embryonic day 18 and increasing dramatically in concentration after the third postnatal week.

A large collaborative study to identify endogenous ligands for orphan G-protein-coupled receptors (GPCRs) discovered the peptides independently [9]. This group referred to the peptides as orexins because they stimulated acute food intake when

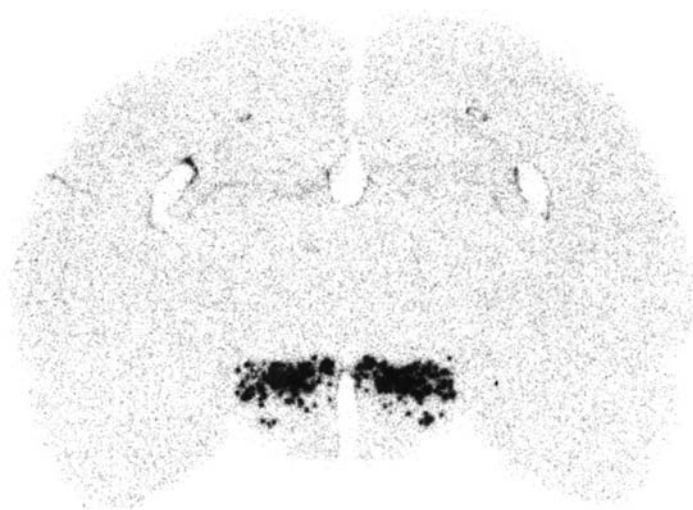


Figure 4.1 First glimpse of hypocretin system. *In situ* hybridization of rat coronal section with complementary DNA (cDNA) isolated in subtractive hybridization study detecting a few thousand neurons in the dorsal–lateral hypothalamus [7].

administered to rats during the daytime. In this chapter, we will refer to the peptides by their first-used name, the hypocretins, but the terms are interchangeable and are both used extensively in the large literature that has grown up around the peptides.

The sequences of the rat [569 non-poly(A) nucleotides] and homologous mouse (582 nucleotides) mRNAs each encodes a 130-residue putative secretory protein with an apparent signal sequence and two additional phylogenically conserved sites for potential proteolytic maturation followed by modification of the carboxy-terminal glycines by peptidylglycine α -amidating monooxygenase [8]. These features suggested that the product of this hypothalamic mRNA served as a preprohormone for two C-terminally amidated, secreted peptides. One of these, hypocretin 2 (Hcrt2, or OxB), was, on the basis of the putative preprohormone amino acid sequence, predicted to contain precisely 28 residues. The other, hypocretin 1 (Hcrt1, or OxA), had a defined predicted amidated C-terminus but, because of uncertainties as to how the amino terminus might be proteolytically processed, an undefined N-terminal extent [8]. The C-terminal 19 residues of these two putative peptides shared 13 amino acid identities, suggesting that the peptides had related structures and functions. This region of Hcrt2 contained a seven-amino-acid match with secretin. Antisera generated against synthetic peptides corresponding to regions of the deduced prohypocretin sequence and to bacterially expressed preprohypocretin have been generated [8, 10–12]. The antisera are specific for the hypocretin-related peptides and have been used extensively to characterize the protein in both anatomical and enzyme-linked immunosorbent assay (ELISA)–type studies, described below.

The detection of the two hypocretin peptides within the brain allowed the exact structures of these endogenous peptides to be determined by mass spectroscopy [9]. The sequence of endogenous Hcrt2, RPGPPGLQGRLQRLQLQANGNHAA GILTM-amide, was the same as that predicted from the cDNA sequence. The N-terminus of Hcrt1 was found to correspond to a genetically encoded glutamine that was derivatized as pyroglutamate. Hcrt1 (33 residues: *EPLPDCCRQKTCSCRL YELLHGAGNHAAAGILTL-amide) contains two intrachain disulfide bonds. Human Hcrt1 is identical to the rodent peptide, whereas human Hcrt2 differs from rodent Hcrt2 at two residues [9].

The mouse hypocretin gene, *HCRT*, is located on chromosome 11, and the human *HCRT* gene maps to chromosome 17q21–q24. Genes that encode conserved preprohypocretins have been detected in pufferfish and frog species, suggesting that the gene arose early in the chordate lineage [13]. Sequence similarities with various members of the incretin family, especially secretin, suggest that the preprohypocretin gene was formed from the secretin gene by three genetic rearrangements: first, a duplication of the secretin gene; second, deletions of the N-terminal portion of the 5' duplicate and the C-terminal portion of the 3' duplicate to yield a secretin with its N- and C-termini leap frogged (circularly permuted); and third, a further duplication of the permuted gene, followed by modifications, to form a secretin derivative that encoded two related hypocretin peptides [13].

Consistent with the hypothesis that the hypocretins and secretin are phylogenically related, portions of their three-dimensional solution structures, as determined by nuclear magnetic resonance, are similar despite their leap-frogged primary sequence, consisting of two adjacent α helices (6–7 and 9–14 amino acids long) separated by a short 2–3-amino-acid turn [14–16]. The longer helix corresponds to the region of identity between the two peptides.

4.3 TWO HYPOCRETIN RECEPTORS

Sakurai and collaborators [9] prepared transfected cell lines stably expressing each of 50 orphan GPCRs and then measured calcium fluxes in these cell lines in response to fractions from tissue extracts. One of these transfected cell lines responded to a substance in a brain extract. Mass spectroscopy showed that this substance was a peptide whose sequence was later identified as that of endogenous Hcrt1. The initial orphan GPCR, Hcrtr1 (also referred to as OX1R), bound Hcrt1 with high affinity but Hcrt2 with 100- to 1000-fold lower affinity. A related GPCR, Hcrtr2 (OX2R), sharing 64% identity with Hcrtr1, was identified by searching database entries with the Hcrtr1 sequence, had a high affinity for both Hcrt2 and Hcrt1 [9]. These two receptors are highly conserved (95%) across species. Radioligand binding studies and calcium flux measurements have shown Hcrt1 to have equal affinity for Hcrtr1 and Hcrtr2, whereas Hcrt2 has ~10-fold greater affinity for Hcrtr2 than for Hcrtr1 [17].

4.3.1 Agonists and Antagonists

Substitution of alanine for leucine at position 11 of human Hcrt2 ([A¹¹]Hcrt2) produces a modified agonist 100-fold more selective than native Hcrt2 for Hcrtr2 over Hcrtr1 [18]. Several Hcrt2 analogs with > 1000-fold selectivity for Hcrtr2 and a truncated form of Hcrt1 (residues 2–23) with modest Hcrtr1 preference have also been produced [19].

SB-334867 [1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea] is an antagonist that has an affinity of 40 nM for Hcrtr1 and is more than 50-fold selective over Hcrtr2 and other GPCRs and ion channels [20–22]. SB-408124 [1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea] is slightly more potent and has greater Hcrtr1 selectivity than SB-334867 [17]. Both compounds are systemically bioavailable and brain penetrant. An Hcrtr1-selective radioligand antagonist has also been described [23]. *N*-Acyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was the first Hcrtr2-selective antagonist [24]. More recently a compound [9,1-(2,4-dibromo-phenyl)-3-((4*S*,5*S*)-2,2-dimethyl-4-phenyl-[1,3]dioxan-5-yl)-urea] has been produced that has a 600-fold selectivity for Hcrtr2 over Hcrtr1 [25].

4.4 HYPOCRETIN CELL BODIES

A few thousand neurons highly positive for Hcrt mRNA and immunoreactivity are located between the rat fornix and the mammillothalamic tracts [7, 8, 10, 26, 27]. These are first detected at embryonic day E18 [28]. Beginning at E20, hypocretin antisera detect a prominent network of axons that project from these cells to other neurons in the perifornical and posterior hypothalamus. Both mRNA and peptide expression diminish after one year of age [29]. The human lateral hypothalamus contains 50,000–80,000 hypocretin neurons [30]. With the exception of one report on Hcrt in the enteric nervous system [31], there is no other place in the brain or periphery where Hcrt-producing neuronal perikarya have been found. Hcrt neurons are 20–30 μm in diameter and are multipolar or fusiform in shape, with two to four primary dendrites bearing few spines. In addition to rats, mice, and humans, Hcrt

neurons with a similar restricted hypothalamic distribution have been detected in monkey, hamster, cat, sheep, pig, chicken, various amphibians, and zebrafish.

The LH contains a collection of neurons that express MCH, a peptide that has been implicated in feeding-related behavior [32]. MCH and hypocretin neurons are distinct but spatially intermingled, each set with a different topological distribution [10, 26, 27, 33]. Neither population is cholinergic, but both populations express acetylcholinesterase [34]. There is a nearly one-to-one correspondence between LH neurons that express the opioid receptor agonist dynorphin and the hypocretin neurons [35], and nearly all Hcrt neurons express secretogranin II [36]. Glutamate, the excitatory amino acid transporter EAAT3, and the vesicular glutamate transporters VGLUT1 and VGLUT2 are expressed by Hcrt neurons [37–41]; thus, Hcrt neurons are likely to be glutamatergic. Other proteins detected in Hcrt neurons include the γ -aminobutyric acid (GABA) type A receptor ϵ subunit, serotonin 5-HT_{1A} receptor, μ -opioid receptor, pancreatic polypeptide Y4 receptor, adenosine A1 receptor, leptin receptor, precursor protein convertase, signal transduction and activator of transcription 3 (STAT-3), and the neuronal activity-regulated pentraxin (NARP), implicated in clustering of ionotropic glutamate receptors [33, 42–49].

4.4.1 Hcrt Projections

Projections from Hcrt-immunoreactive cell bodies are detected throughout the brain, with the highest density of terminal fields seen in the hypothalamus [8, 10, 27]. Hypothalamic regions receiving projections include the LH and posterior hypothalamic areas (regions of Hcrt and MCH neuronal populations), the dorsomedial hypothalamus (DMH), the paraventricular hypothalamic nucleus, and arcuate nucleus. Hcrt is reciprocally connected with neuropeptide Y (NPY) and leptin receptor–positive neurons in the arcuate nucleus [50], an area important in feeding behaviors and endocrine regulation. Hcrt neurons also make reciprocal synaptic contact with neighboring MCH neurons [51, 52].

Prominent Hcrt fibers project from the LH to apparent terminal fields in many areas of the brain. Peyron and colleagues [10] referred to four Hcrt efferent pathways: dorsal and ventral ascending pathways and dorsal and ventral descending pathways. The dorsal ascending pathway projects through the zona incerta to the paraventricular nucleus of the thalamus, central medial nucleus of the thalamus, lateral habenula, substantia innominata, bed nucleus of the stria terminalis, septal nuclei, dorsal anterior nucleus of the olfactory bulb, and cerebral cortex. The ventral ascending pathway projects to the ventral pallidum, vertical and horizontal limb of the diagonal band of Broca, medial part of the accumbens nucleus, and olfactory bulb. The dorsal descending pathway projects through the mesencephalic central gray to the superior and inferior colliculi and the pontine central gray, locus coeruleus (LC), dorsal raphe nucleus, and laterodorsal tegmental nucleus. A second bundle of fibers projects through the dorsal tegmental area to the pedunculopontine nucleus, parabrachial nucleus, subcoeruleus area, nucleus of the solitary tract, parvocellular reticular area, dorsal medullary region, and caudal spinal trigeminal nucleus. This tract continues to all levels of the spinal cord [11]. The ventral descending pathway runs through the interpeduncular nucleus, ventral tegmental area (VTA), substantia nigra pars compacta, raphe nuclei and reticular formation, gigantocellular reticular nuclei, ventral medullary area, raphe magnus, lateral

paragigantocellular nucleus, and ventral subcoeruleus. The cumulative set of projections is consistent with the combined patterns of expression of the two hypocretin GPCRs (discussed below). Although a large proportion of Hcrt neurons contribute projections to multiple terminal fields, various subgroups of cells make preferential contributions to particular fields [53, 54]. The projection fields in humans are comparable to those in rodents [30]. The diffuse nature of Hcrt projections provided the first evidence of the potential for multiple physiological roles for the peptides.

4.4.2 Hcrt at Synapses

Electron microscopic examination revealed that hypocretin immunoreactivity is associated with the rough endoplasmic reticulum, the Golgi network, and cytoplasmic dense-core vesicles [8, 10, 50]. The latter have been observed along myelinated axons and at presynaptic boutons apposed to dendritic shafts in both the periaqueductal gray and the LC, where the synapses are with tyrosine hydroxylase-positive noradrenergic dendrites. The accumulation of the hypocretins within dense-core vesicles at axon terminals suggested that they might have intercellular signaling activity. In the brain stem and spinal cord of cats, age-dependent enlargement of hypocretin synapses has been observed, suggesting age-related changes in Hcrt transmission [55].

4.4.3 Hcrt Afferents

In addition to reciprocal connections with the MCH neurons of the LH and with NPY/agouti-related peptide neurons and galaninlike peptide neurons [56] of the arcuate, corticotropin-releasing factor (CRF)-positive inputs arising from the paraventricular hypothalamic nucleus have been detected in close apposition to Hcrt perikarya [54]. Hypocretin neurons receive direct projections positive for arginine-vasopressin and vasoactive intestinal peptide from neurons in the suprachiasmatic nucleus (SCN) [37], which is responsible for generating the circadian rhythm, and also innervation from the DMH, which itself responds to SCN input [57].

Hcrt neurons are predominantly controlled by local glutamatergic excitatory interneurons [39]. Perikaryal GABA input onto the Hcrt neurons is very minimal, approximately 10% that of the glutamatergic input. Other afferents include brain stem noradrenergic and dorsal raphe nucleus serotonergic inputs as well as cholinergic afferents [39, 58].

4.4.4 Receptor Distributions

The mRNAs that encode the two hypocretin receptors and the receptor proteins themselves, detected by immunohistochemistry, are both enriched in the brain and moderately abundant in the hypothalamus but have different distributions within the brain [59–61]. Hcrt1 mRNA is prominent in the prefrontal and intralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus, LC, laterodorsal and pedunculopontine tegmental nuclei (at higher density than Hcrt2), pontine raphe, raphe magnus, raphe obscurus, dorsal motor vagal complex, and spinal cord. Additional forebrain regions expressing moderate to high levels of Hcrt1 mRNA are the tenia tecta, bed nucleus of the stria

terminalis, horizontal limb of the diagonal band of Broca, and medial amygdala. Immunoreactive terminals have been detected in the LH. This receptor has also been detected in the retina [62].

Hcrtr2 mRNA (there are two alternative splice forms, each encoding proteins with slightly different structure and anatomical distribution but no demonstrated functional differences) [63] is detected in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and various nuclei of the hypothalamus, including the tuberomammillary nucleus, dorsomedial nucleus, paraventricular nucleus, ventral premammillary nucleus, ventral periaqueductal gray, midbrain reticular formation, dorsal interpeduncular nucleus, Barrington's nucleus, sensory trigeminal nucleus, ventrolateral medulla, and dorsal vagal nucleus. Hcrtr2 mRNA is prominent in the medial septum, vertical and horizontal limbs of the diagonal band of Broca, substantia innominata, and cortical amygdala.

Both receptors are detected in dorsal raphe, the VTA, paraventricular thalamic nucleus, and intergeniculate leaflet, with lesser density in the rhomboid, reunions, and other midline nuclei. In the cerebral cortex, Hcrtr1 mRNA is expressed primarily in layers II, III and V, whereas Hcrtr2 mRNA is found at higher density in layers II and VI and more diffusely in other layers. In the hippocampus, Hcrtr1 is expressed mainly in the CA2 region and medial dentate gyrus, while Hcrtr2 was most abundant in CA3 [64].

The distribution of Hcrtr receptors is largely consistent with Hcrtr axon innervation patterns. The composite distribution of the two Hcrtr receptors strongly resembles the distribution of the MCH receptor [65]. In the LC, amygdala, and other brain stem noradrenergic groups, MCH receptor mRNA distribution is similar to that of the Hcrtr1. In regions such as the septum, hypothalamus, and much of the brain stem, the distribution of MCH receptor mRNA resembles that of the Hcrtr2 [65].

The Hcrtr receptors are not restricted to the central nervous system (CNS): In the periphery, they are widely expressed, especially in endocrine tissues. Hcrtr1 or Hcrtr2 have been detected in the pituitary, adrenal gland, testis, gastrointestinal tract, pancreas, and pineal gland.

4.5 HYPOCRETINS: EXCITATORY NEUROTRANSMITTERS

Three observations suggested that the hypocretins might possess neurotransmitter activities: (i) the detection of Hcrtr neurons in a specialized region of the hypothalamus, (ii) the processing of the precursor into two related peptides, and (iii) the detection of the peptides in vesicles at synapses. Bath application of synthetic Hcrtr2 to mature hypothalamic neurons evoked increases in the frequency of postsynaptic currents [8]. Hypocretin-mediated excitation has been found in a large number of brain regions, many of them involved in arousal. Hypocretin increases activity in the hypothalamus [66, 67], LC [49, 68, 69], dorsal raphe [70, 71], dorsal horn of the spinal cord [72], spinal motoneurons [73], nucleus of the solitary tract [74], dorsal motor nucleus [75], dorsolateral tegmentum [76], tuberomammillary nucleus [77, 78], basal forebrain [79, 80], neocortex [81], hippocampus [82], midline thalamus [83], and trigeminal nucleus [84].

Hcrtr2 has a potent effect at both presynaptic and postsynaptic receptors [66]: In the presence of tetrodotoxin, the hypocretins increase the frequency, but not the

amplitude, of miniature postsynaptic currents (presynaptic effect) and evoke an increase in cytoplasmic calcium by opening plasma membrane Ca^{2+} channels in arcuate postsynaptic neurons (postsynaptic effect). Most synaptic activity in hypothalamic circuits is attributable to axonal release of GABA or glutamate. Hypocretin, acting directly at axon terminals, can increase the release of each of these amino acid transmitters, as demonstrated by whole-cell patch-clamp recording [66].

Both Hcrt1 and Hcrt2 evoke rises in Ca^{2+} , as measured by fura-2 imaging, in about one-third of hypothalamic neurons, probably by opening a transient receptor potential channel (TRPC)-type calcium channel [85–87]. Responses to hypocretin are completely blocked by the protein kinase C-specific inhibitor bisindolylmaleide and by phospholipase C (PLC) inhibitors [66], suggesting that the hypocretins work through a family of guanosine triphosphate (GTP) binding proteins (G_q) that activate protein kinase C (PKC) and mobilization of intracellular calcium. G_q -activated signaling cascades result in phosphorylation of Ca^{2+} channels, which can increase Ca^{2+} conductance and neuronal excitability [88, 89]. The nonamidated forms of the peptides are not electrophysiologically active [88]. In the substantia nigra pars reticulata, hypocretin stimulation was sensitive to an inhibitor of protein kinase A, which mediates effects of cyclic adenosine monophosphate (cAMP), but insensitive to blockers of PKC [90], implicating a role of the G_s system.

The adrenal glands also express Hcrt2 [91, 92]. Treatment of human adrenal membranes from fetal or adult tissue with Hcrt1 increased the labeling of G_s and G_i in both preparations and additionally G_q in the adult preparation. Thus, although the majority of hypocretin signaling is excitatory, it may be inhibitory in some cases [93]. Acting as excitatory peptides, the hypocretins can enhance the activities of both excitatory and inhibitory neurons.

4.6 FEEDING AND METABOLISM

Sakurai and colleagues [9] found that intracerebroventricular (ICV) administration of either Hcrt1 or Hcrt2 increased short-term food consumption in rats. Furthermore, rats that had been deprived of food for 48 h showed increased concentrations of hypocretin mRNA and peptides in the hypothalamus [9, 94]. Feeding responses can be elicited by local administration of Hcrt1 to the paraventricular nucleus, the dorsomedial nucleus, the lateral hypothalamus, or the perifornical area [95]. ICV administration of Hcrt2 also increases food intake in sheep [96] and goldfish [97].

Many observations leave little doubt that the hypocretin system influences and is influenced by primary nutritional homeostasis circuits, but other findings suggest that the hypocretins are not critical players in feeding activities but rather play roles in increasing arousal and motivation levels so that feeding can take place. Hypocretin-immunoreactive fibers form synapses on neurons in the arcuate nucleus that contain NPY, an important orexigenic (appetite-stimulating) peptide, and with pro-opiomelanocortin (POMC)-expressing neurons, which produce α -melanocyte-stimulating hormone, a satiety factor [26, 27, 50]. Hypocretin neurons express leptin receptors [33, 50, 98], and preprohypocretin mRNA expression is reduced in *obese* (*ob/ob*) mice [99], which lack leptin. Hypocretin neurons receive inputs from NPY- and agouti-related peptide (AgRP)-positive neurons in the arcuate nucleus, which themselves express leptin receptors, and NPY stimulates *c-fos* expression by

hypocretin neurons, whereas NPY receptor antagonists block the feeding effect of hypocretins [100, 101]. Administration of an antiserum directed against Hcrt1 attenuates the feeding response to ICV NPY, and pharmacological blockade of the NPY receptor reduces the feeding stimulatory effect elicited by Hcrt1 [100–102]. Hcrt-expressing cells respond to circulating leptin by reducing Hcrt1 concentrations and *c-fos* expression [102–104]. Also supporting the idea that the hypocretin neurons are involved in feeding is the observation that they express STAT3, a transcription factor that is induced by leptin [33]. However, whereas NPY-induced feeding is completely inhibited by leptin, the Hcrt feeding response is only partially suppressed by leptin [105]. Cultured neurons isolated from the arcuate nucleus show either of two responses to the Hcrts [106]. In neurons immunoreactive for NPY, intracellular Ca^{2+} is increased in response to Hcrt1 (more so than Hcrt2), and the increase is blocked by PLC, PKC, and calcium uptake inhibitors, indicative of signaling through Hcrt1 via PLC, PKC, and inositol-1,4,5-triphosphate (IP_3) pathways. In POMC-immunoreactive neurons, the two peptides equipotently decreased Ca^{2+} by a pertussis toxin-sensitive mechanism, indicative of signaling through Hcrt2 via G_i/G_o pathways. The peptides also decreased Ca^{2+} in glucose-responsive neurons in the ventromedial hypothalamus. These three effects are reciprocal to those induced by leptin [106].

Hcrt cells are sensitive to glucose and food deprivation: The activity of hypocretin neurons and their expression of hypocretin mRNA and *c-fos* increase during hypoglycemia and after injection of Intralipid; hypocretin mRNA decreases during glucopenia; and *c-fos* expression increases during fasting [107–112]. Acute administration of the Hcrt1-selective antagonist SB-334867 suppresses food intake in rats. Interestingly, SB-334867 also reduced weight gain for 3–5 days posttreatment [113–115], despite being no longer detectable after 12 h, and repeated administration of the antagonist reduced the food intake and weight gain over 14 days in *ob/ob* mice [116]. These results are consistent with a complex circuitry of appetite-controlling signaling molecules in the arcuate and lateral hypothalamus in which hypocretin might play a role.

Not all data support this notion, though, particularly when the other activities of the hypocretins are considered (see below). Hcrt1-induced increases in food intake were small relative to those induced by NPY infusion [117, 118]. Others have measured no alteration in Hcrt1 peptide concentration or Hcrt mRNA in the rat hypothalamus in response to either fasting or a high-fat diet, and no effect on Hcrt mRNA of experimentally induced diabetes [119–121]. However, Hcrt mRNA increases after leptin administration to fasted mice and increases in response to a high-fat diet [122]. Hcrt1 stimulated acute food consumption in 4-month-old rats but not 25-month-old rats [123] and substantially decreased food intake 2 h after central administration to rhesus monkeys, consistent with an anorectic action [124].

It is difficult to attribute physiological effects to ICV administration of high doses of hypocretin, which might activate circuits other than those that would be activated by local axonal release of the transmitter [66]. And, the Hcrts might be orexigenic only under some physiological states (perhaps related to circadian rhythms or stress). In this regard, it might be significant that the hypocretins activate dopamine-mediated stereotypic behaviors. Hcrt1 peptide concentrations in the hypothalamus are under circadian control and are highest during the awake, dark period in nocturnal rodents [125]. During fasting, Hcrt1 accumulation in the cerebrospinal

fluid (CSF) does not exceed concentrations normal for the waking period, suggesting that some of the food uptake effect may result from arousal rather than direct feeding pressure [126]. Continuous administration of Hcrt1 for seven days in rats does not significantly alter daily food intake, body weight, blood glucose, total cholesterol, or free fatty acid levels [127], suggesting that many of hypocretin's effects may be limited to short-term, immediate stimulation of feeding behavior due to increased wakefulness. That is, animals eat more and are motivated to eat when they are awake. Consistent with these views, although most circadian parameters and behaviors are unaffected in mice in which the Hcrt neurons have been ablated (mice discussed below), increases in wakefulness and locomotor activities that normally precede an anticipated meal during a restricted feeding regimen are greatly diminished in the ablated mice [128–130].

4.7 AUTONOMIC AND ENDOCRINE EFFECTS

Hypocretin neurons receive inputs from brain stem areas that are associated with cardiovascular function and project to the ventrolateral medulla, the LC, the lateral paraventricular nucleus, the nucleus of the solitary tract, the pre-Bötzinger region of the ventrolateral medulla, phrenic motoneurons, and other areas that have been implicated in the regulation of blood pressure, heart rate, and breathing [131, 132]. Projections to the arcuate nucleus also suggested a role in the regulation of hormone release. In the ovine hypothalamus, there are hypocretin terminals on the neurons that produce gonadotropin-releasing hormone, suggesting that hypocretin might particularly modulate reproductive endocrinology [133]. In addition, projections to the raphe magnus and subcoeruleus suggested a role for hypocretins in the regulation of body temperature. The dense hypocretinergic projections to the ventrolateral preoptic area, tuberomammillary nucleus (TMN), pontine reticular formation, pedunculopontine (PPT) and laterodorsal tegmental (LDT) area, and LC suggested involvement in states of arousal [10, 68, 134]. Very strong hypocretin-immunoreactive projections have been described in regions of the spinal cord that are related to modulation of pain [11], and hypocretin-like immunoreactivity has also been detected in the intestinal epithelium [31].

In accordance with the wide distribution of hypocretin terminals, ICV administration of the hypocretins affects not only feeding but also several other functions. Both Hcrt1 and Hcrt2 elevate mean arterial blood pressure, heart rate (both suppressed by SB334867), and oxygen consumption [135–138]. Microperfusion of the Hcrts in pre-Bötzinger or phrenic motoneuron sites increases diaphragm muscle activity [132]. Hcrt1 increases body temperature independent of peripheral thermogenesis, at least in part via clozapine-sensitive pathways in the cerebral cortex [139–142]. Hcrt1 also increases water consumption and stimulates gastric acid secretion in the gut [143, 144] and increases locomotor activity and wakefulness while decreasing slow-wave and depressing rapid-eye-movement (REM) sleep [68, 69, 145–147].

The peptides also stimulate the secretion of luteinizing hormone in ovariectomized and proestrus female rats (suppressed by central administration of SB334867) and hypothalamic explants of male pituitaries [148, 149]. Consistent with these findings, in humans, LH concentration in serum and pulsatile LH secretion are lower in male narcolepsy patients (who lack hypocretin function; see below) than in unaffected

controls [150]. *Hcrtr* concentrations are elevated during proestrus in cycling female rats but not noncycling middle-aged rats and in pregnant rats, and *c-fos* is increased in *Hcrtr* neurons in lactating mice [151–153]. In male rats, *Hcrtr1* increased basal testosterone secretion [154]. *Hcrtr1* decreases the concentrations of circulating growth hormone and prolactin while increasing corticosterone, adrenocorticotrophic hormone (ACTH), and insulin levels [68, 155–159]. Hypoglycemia-induced elevation of pancreatic nerve firing is antagonized by SB334867 microinjection in the dorsal motor nucleus of the vagus, indicative of a central circuit in this pathway [160]. *Hcrtr2*, but not *Hcrtr1*, increases circulating thyroid-stimulating hormone [161] and has direct effects on the pituitary, adrenal, and pineal glands [91, 162, 163].

Both peptides depolarize CRF neurons in the paraventricular hypothalamic nucleus (PVN) and increase CRF, *c-fos*, and arginine vasopressin mRNA concentrations in the PVN and thus have clear effects on the hypothalamic–pituitary–adrenal (HPA) axis and stress-related physiology; various stress paradigms increase *c-fos* expression by *Hcrtr* neurons [67, 164–168]. *Hcrtr2* is directly excitatory on superficial dorsal horn neurons of the spinal cord [71] and exhibits an analgesic effect in models of pain [169]. There are several examples in which either *Hcrtr1* or *Hcrtr2*, but not both peptides, effects a response, suggesting that the two peptides are not redundant. In some cases, the effects can be explained by differential involvement of *Hcrtr1* or *Hcrtr2*; in others, differential resistance of the peptides to degradation may provide the explanation.

4.8 MOTIVATION AND ADDICTION

Hcrtr neurons are highly responsive to morphine and are activated by naltrexone-precipitated withdrawal. However, the response of these neurons is heterogeneous, suggesting that there might be different populations of *Hcrtr* cells [170]. The expression of the *Hcrtr* gene increases only after precipitated withdrawal. The neurons express the μ -opioid receptor; hence their response may be due to direct actions of morphine and naltrexone [44]. These observations might explain why animals self-administer heroin to the LH [171]. *Hcrtr* knockout mice exhibit dramatically attenuated morphine withdrawal symptoms [44]. *Hcrtr* neurons have extensive projections to the mesolimbic dopamine and noradrenergic (LC) pathways, regions well studied for their roles in drug addiction. These neurons also project to and inhibit nucleus accumbens neurons [93].

Rats can be trained to turn a wheel to deliver electrical current to the LH: LH self-stimulation (LHSS). LHSS thresholds measure brain reward systems; lower thresholds represent increased reward. Most drugs of abuse lower LHSS thresholds. LHSS is thought to be rewarding in part because it activates cholinergic neurons in the LDT and the PPT nuclei that consequently activate dopaminergic neurons in the VTA [172]. Hypocretins excite LDT cholinergic neurons both directly and indirectly acting synergistically with glutamatergic afferents [76] to drive dopamine release in the nucleus accumbens by exciting dopaminergic neurons in the VTA. Thus, the *Hcrtr* system acts as a modulator of brain reward function. Interestingly in this regard, naltrexone injected into the accumbens suppresses the *Hcrtr1*-induced acute feeding response, suggesting that the response is mediated by opiodergic pathways involved in motivation [173].

4.9 PAIN AND ANAESTHESIA

In models of pain elicited by noxious stimuli or chronic constriction, Hcrt administered intrathecally or microinjected into the posterior hypothalamus decreased pain parameters [174, 175]. Hypocretin knockout mice (see below) exhibited greater hyperalgesia and less stress-induced analgesia than wild-type mice [176]. Hcrt1 decreases barbiturate anaesthesia time in rats by 15–40%, an action reversed by SB-334867 [177]. *In vitro*, barbiturates inhibit Hcrt-induced norepinephrine release, although they do not interact directly with Hcrt receptors. In isoflurane-anesthetized animals, Hcrt1 elicits arousal without cardiovascular activation, in contrast to its effect on awake animals [178]. Pain and stress activate Hcrt neurons, which respond by inhibiting pain pathways.

4.10 HIPPOCAMPAL PLASTICITY

In vivo studies have shown that Hcrt enhances long-term potentiation in the dentate gyrus induced by high-frequency stimulation in the perforant path [179]. Paradoxically, ICV administration of hcr1 in rats impairs spatial learning in the Morris water maze [180].

In vitro recordings have demonstrated that Hcrt1 can induce long-term potentiation (LTP) at Schaffer collateral CA1 but not mossy fiber CA3 synapses [82]. Pharmacological analysis revealed that Hcrt-induced LTP requires coactivation of ionotropic and metabotropic glutamatergic, GABAergic, as well as noradrenergic and cholinergic receptors. Hcrt may also affect hippocampal physiology indirectly by increasing release of norepinephrine [181]. These data suggest that Hcrt may be involved in regulating the threshold and weight of synaptic connectivity, providing a mechanism for integration of multiple transmitter systems [82]. Thus, the Hcrt also participate in some aspects of metaplasticity.

4.11 NARCOLEPSY: DISEASE OF HYPOCRETIN SYSTEM

Sleep is characterized by complex patterns of neuronal activity in thalamocortical systems [182–184]. The fast, low-amplitude electroencephalogram (EEG) activity of the aroused state is replaced by synchronized high-amplitude waves that characterize slow-wave sleep. This pattern develops further into high-frequency waves that define paradoxical, or REM, sleep. Switching among these states is controlled in part by the activities of neurons in the hypothalamic ventrolateral preoptic nucleus and a series of areas referred to as the ascending reticular activating system, which is distributed among the PPT and LDT, LC, dorsal raphe nucleus (DRN) and TMN and regulates cortical activity and arousal [185]. The balance struck among the various phases of sleep and the rapid transitions from one phase to the next are determined by requirements for wakeful activities, homeostatic pressures for sleep, and circadian influences [186, 187].

The first case of human narcolepsy was reported in 1877 by Westphal, and the sleep disorder acquired its name from Gélinau in 1880. Narcolepsy affects around 1 in 2000 adults, appears between the ages of 15 and 30 years, and shows four

characteristic symptoms: (i) excessive daytime sleepiness with irresistible sleep attacks during the day; (ii) cataplexy (brief episodes of muscle weakness or paralysis precipitated by strong emotions such as laughter or surprise); (iii) sleep paralysis, a symptom considered to be an abnormal episode of REM sleep atonia, in which the patient is suddenly unable to move for a few minutes, most often upon falling asleep or waking up; and (iv) hypnagogic hallucinations, or dreamlike images that occur at sleep onset. These latter symptoms have been proposed as pathological equivalents of REM sleep. The disorder is considered to represent a disturbed distribution of sleep states rather than an excessive amount of sleep.

Studies with monozygotic twins have shown that narcolepsy is weakly penetrant: In only 25% of cases does the monozygotic twin of an affected individual also develop the disorder. Sporadic narcolepsy (which accounts for 95% of human cases) is highly correlated with particular class II human leukocyte antigen (HLA) DR and DQ histocompatibility haplotypes in about 90% of patients, but most people with these haplotypes are not narcoleptic [188]. Because many autoimmune disorders are HLA linked and because of the late and variable age of disease onset, narcolepsy has long been considered a likely autoimmune disorder, but the targets of the immune attack were not known.

Both sporadic and heritable narcolepsy are observed in dogs, and the symptoms resemble those exhibited by human narcoleptics. The first link between the hypocretins and narcolepsy came from genetic linkage studies in a colony of Doberman pinschers in which narcolepsy was inherited as an autosomal recessive, fully penetrant phenotype. Fine mapping and cloning of the defective canine narcolepsy gene showed it to be the gene that encodes the hypocretin receptor *Hcrtr2* [189]. The mutation in the Doberman lineage is an insertion of a short interspersed repeat (SINE element) into the third intron of *HCRT2* that causes aberrant splicing of the *Hcrtr2* mRNA (exon 4 is skipped) and results in a truncated receptor protein. In cells that have been transfected with the mutant gene, the truncated *Hcrtr2* protein does not properly localize to the membrane and, therefore, does not bind its ligands [190]. Analysis of a colony of narcoleptic Labradors revealed that their *HCRT2* gene contained a distinct mutation that resulted in the skipping of exon 6, also leading to a truncated receptor protein. A third family of narcoleptic Dachshunds carries a point mutation in *HCRT2* that results in a receptor protein that reaches the membrane but cannot bind the hypocretins. Genetically narcoleptic dogs have increased CSF levels of Hcrtr, which diminishes until symptoms appear at four weeks, then increases [191]. Administration of immunoglobulins or immunosuppressive/anti-inflammatory drugs doubles time to symptom onset and severity of symptoms, suggesting that *HCRT2* deficits alone are not sufficient to elicit all of the symptomatology initiated by the loss-of-function mutations [192, 193].

In knockout mice in which the hypocretin gene was inactivated by homologous recombination in embryonic stem cells, continuous recording of behavior revealed periods of ataxia which were especially frequent during the dark period [194]. EEG recordings showed that these episodes were not related to epilepsy and that the mice suffered from cataplectic attacks, a hallmark of narcolepsy. In addition, the mutant mice exhibited increased REM sleep during the dark period as did their wild-type littermates, and their EEGs showed episodes of direct transition from wakefulness to REM sleep, another event that is unique to narcolepsy. Waking and non-REM sleep bouts were brief, with more transitions among all three states, suggestive of a

behavioral state instability with low state transition thresholds [195]. Mice with an inactivated *HCRT* gene have a milder narcoleptic phenotype than the *HCRT* knockouts; *HCRT1* knockouts exhibit only a sleep fragmentation phenotype, whereas double *HCRT1* and *HCRT2* mutants recapitulate the full *HCRT* knockout phenotype [196], suggesting that signaling through both receptors contributes to normal arousal, although the role of *HCRT2* is greater than that of *HCRT1*. Similar observations were made in rats in which the hypocretin neurons of the lateral hypothalamus were inactivated by saporin targeting [197], although in this model cataplexy was not observed. However, in mice [198], or rats in which the hypocretin neurons are ablated due to the expression of the toxic ataxin-3 fragment from the *Hcr* promoter, *Hcr* neurons are lost at 17 weeks, and the hallmarks of narcolepsy ensue, including episodes of muscle atonia and loss of posture resembling cataplexy [199].

Nishino and colleagues [200] studied hypocretin concentrations in the CSF of normal controls and patients with narcolepsy by radioimmunoassay. In control CSF, hypocretin concentrations were highly clustered, suggesting that tight regulation of the substance is important. However, of nine patients with narcolepsy, only one had a hypocretin concentration within the normal range. One patient had a greatly elevated concentration, while seven patients had no detectable circulating hypocretin. In an expanded study, hypocretin was undetectable in 37 of 42 narcoleptics and in a few cases of Guillain–Barré syndrome [201]. CSF hypocretin was in the normal range for most neurological diseases but was low, although detectable, in some cases of CNS infections, brain trauma, and brain tumors. Low CSF hypocretin concentrations have also been measured in a patient with acute disseminated encephalomyelitis presenting similarities to von Economo’s encephalitis lethargica which returned to the normal range as daytime sleepiness was reduced [202] and in two patients with Prader–Willi syndrome accompanied by excessive daytime sleepiness (EDS) [203].

Peyron, Thannikal, and collaborators [204, 205] found that, in the brains of narcolepsy patients, they could detect few or no hypocretin-producing neurons. Whether the hypocretin neurons are selectively depleted, as is most likely, or only no longer expressing hypocretin is not yet known, although one report showed some indications of gliosis [205]. The codistributed MCH neurons were unaffected. Furthermore, a single patient with a non-HLA-linked narcolepsy carries a mutation within the hypocretin gene itself. The mutation results in a dominant-negative amino acid substitution in the secretion signal sequence that sequesters both the mutant and heterozygous wild-type hypocretin nonproductively to the smooth endoplasmic reticulum [204]. Amino acid substitutions in *Hcrtr2* have been found in two EDS patients and one Tourette’s syndrome patient: In each case the variant receptor exhibited reduced response to high concentrations of *Hcr* [206].

These findings leave no doubt as to the central role of the hypocretin system in this sleep disorder. Because most cases are sporadic, mutations in the hypocretin gene or those for its receptors can account for no more than a small subset of the human narcolepsies. The HLA association, loss of neurons with signs of gliosis, and age of disease onset are consistent with autoimmune destruction of the hypocretin neurons accounting for the majority of narcolepsy [207], although a non-immune-mediated degenerative process has not been ruled out. For example, studies of hypothalamic slice cultures have revealed that *Hcr* neurons are more sensitive to excitotoxic injury elicited by quinolinic acid than are neighboring MCH neurons, suggesting that

glutamatergic signaling could contribute to their selective loss [208]. Interestingly, hypocretin cell loss has recently been described in Huntington disease (HD) patients [209] and in R6/2 mice, which expresses *exon 1* of the human mutant *HD* gene with ~150 CAG repeats [210]. In advanced stages, these mice display several clinical features reminiscent of HD but relatively little cell death. Thus, Hcrt neurons may have a very low threshold for neuronal apoptosis caused by a variety of environmental stimuli. The narcolepsies as a group are probably a collection of disorders that are caused by defects in the production or secretion of the hypocretins or in their signaling, and these could have numerous genetic, traumatic, viral, and/or autoimmune causes.

Measurement of Hcrt1 in human CSF provides a reliable diagnostic for sporadic narcolepsy. Although local release of Hcrt at its targets within the brain varies during the 24-h day, CSF Hcrt1 levels are relatively stable [125, 211]. In a study of 274 patients with various sleep disorders (171 with narcolepsy) and 296 controls, a cutoff value of 110 pg/mL (30% of the mean control values) was the most predictive of narcolepsy [212]. Most narcolepsy patients had undetectable levels, while a few had detectable but very reduced levels. The assay was 99% specific for narcolepsy.

Hcrt1 has also been detected in plasma, although its origin remains to be demonstrated and high nonspecific background immunoreactivities partially mask its detection. Decreased levels of plasma Hcrt1 were measured in narcoleptic patients using high-performance liquid chromatography separation to confirm that the signal included genuine Hcrt1 [213]. Reductions in daytime plasma Hcrt have been detected in patients with obstructive sleep apneahypopnea syndrome [214, 215].

Given that most human narcolepsy is sporadic and results from depletion of Hcrt-producing neurons, replacement therapies can be envisioned. Small-molecule agonists of the hypocretin receptors might have therapeutic potential for human sleep disorders and might be preferable to the traditionally prescribed amphetamines. ICV administration of Hcrt1 to normal mice and dogs strongly promotes wakefulness [216, 217]. The effect is predominantly mediated by Hcrt2, because the same dose of Hcrt1 has no effect in Hcrt2-mutated narcoleptic dogs [216, 217]. Transgenic expression of preprohypocretin in the brains of mice in which the Hcrt neurons were ablated prevented cataplexy and REM abnormalities, and central administration of Hcrt1 to Hcrt neuron-ablated mice prevented cataplexy and increased wakefulness for 3 h [218]. Hcrt1 has low penetrance of the blood–brain barrier, so a centrally penetrable agonist will need to be devised.

4.12 HYPOCRETIN AND AROUSAL CIRCUITY

Because narcolepsy is the consequence of a defective hypocretin system, it follows that the dominant role of the system is in maintenance of the waking state and suppression of REM entry, and data about the hypocretins give insights as to how this is accomplished (Fig. 4.2). The hypocretin neurons project to various brain stem structures of the ascending reticular activating system which express one or both of the hypocretin receptors and have been implicated in regulating arousal. The noradrenergic neurons of the LC, the serotonergic neurons of the dorsal raphe, and the histaminergic neurons of the TMN are all so-called REM-off cells: Each group fires rapidly during wakefulness, slowly during slow-wave sleep, and hardly at

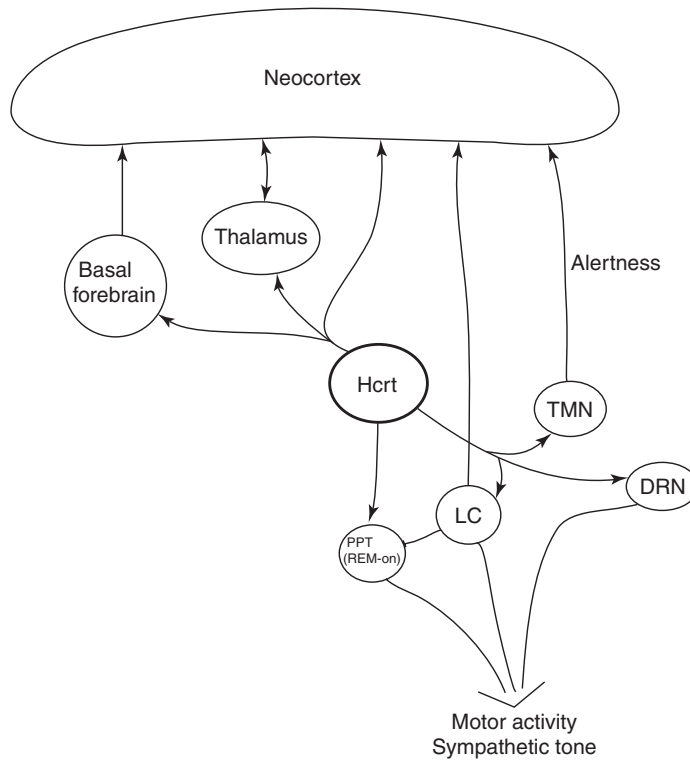


Figure 4.2 Hypocretin projections excite LC and DRN neurons to elevate muscle tone and TMN neurons to promote wakefulness. These components of the ascending reticular activating system, and the Hcrt neurons themselves, project to and stimulate thalamic and basal forebrain neurons, and all of these groups contribute to the depolarization of the cerebral cortex. The LC inhibits REM generator neurons in the PPT, which themselves receive Hcrt input.

all during REM [185, 219]. Each of these structures sends projections to a diverse array of targets in the forebrain, and their firing stimulates cortical arousal. The activity state of these groups of aminergic neurons is one of the features that distinguish wakefulness from REM. Additionally, and importantly, the hypocretin neurons project to other brain areas that have been implicated in arousal. For instance, the hypocretins, acting through Hcrtr2, excite cholinergic neurons of the basal forebrain which produce the cortical acetylcholine characteristic of the desynchronized EEG that is associated with wakefulness and REM [220]. Direct infusion of the hypocretins into the basal forebrain produces dramatic increases in wakefulness [146, 147, 221].

4.12.1 LH Neurons

Among the neurons of the perifornical lateral hypothalamus, 53% increase their firing rates during both wakefulness and REM but decrease their activities during slow-wave sleep [222]. An additional 38% of the neurons in this area are activated

only during the awake phase. The Hcrt neurons are among the latter group; that is, they are REM off. The off state is most likely established and maintained by inhibition by GABA interneurons, as infusion of the GABA_A antagonist bicuculline into the LH of spontaneously sleeping rats increased both wakefulness and *c-fos* expression by Hcrt neurons [223]. Electrophysiological studies on Hcrt neurons, identified in slice culture by their selective transgenic expression of green fluorescent protein and confirmed by appropriate agonists and antagonists, demonstrate that they are hyperpolarized via the action of glutamate (probably originating from local glutamatergic interneurons) acting at group III metabotropic receptors and NPY (from arcuate neurons) acting at Y1 receptors coupled to an inwardly rectifying potassium channel and are depolarized by glucagon-like peptide (GLP) from the brain stem acting through the GLP-1 receptor via a non selective cation conductance [224–227]. Hypocretin neurons express *c-fos* during the waking period (nighttime in rats) and *c-fos* expression is increased by sleep deprivation and methamphetamine [228]. The stimulant modafinil, which is commonly used to treat the drowsiness associated with narcolepsy, greatly elevates *c-fos* expression in hypocretin neurons [194, 229].

Hypocretin levels fluctuate circadianly, being highest during waking, and peptide concentrations increase as a consequence of forced sleep deprivation [125, 211, 230], suggesting that the hypocretins and the activity of the hypocretin neurons serve as pressures that oppose sleep. Interestingly, the amplitude of the circadian oscillation of hypocretin levels is decreased in patients with clinical depression, and treatment with the antidepressant sertraline partially restores the circadian oscillation observed in control subjects [211]. In the absence of environmental light cues, circadian cycling of Hcrt persists, but ablation of the SCN abolished cycling and reduced Hcrt in CSF [231, 232]. In squirrel monkeys, a species that consolidates its waking period into a single bout, limitation of locomotor activity did not have a great effect of CSF hypocretin [233]; however, in the Nile grass rat, a species that varies its distribution of day and night activity in the presence of a running wheel, wheel running affected the activity of Hcrt neurons [234]. In aged rats, peak CSF Hcrt levels are diminished, perhaps leading to increased napping associated with aging [235]. Other perturbations that increase *c-fos* expression in Hcrt cells include treatment with leptin, ghrelin, hypoglycemia, and food and sleep deprivation.

4.12.2 Noradrenergic Systems

The noradrenergic LC neurons fire constantly during wakefulness. In addition to their projections to the forebrain, these neurons send inhibitory projections to cholinergic REM-on (fire during wakefulness and more rapidly during REM but do not fire during slow-wave sleep) generator neurons in the PPT and LDT, which project to the pontine reticular formation [236, 237]. Hypocretin axons form synapses on these LC neurons, which express Hcrtr1 postsynaptically [49, 69]. Local administration of Hcrt1, but not Hcrt2, to the LC suppresses REM in a dose-dependent manner and increases wakefulness [69]. These effects are neutralized by an antibody that prevents binding of Hcrt1 to Hcrtr1. Administration of Hcrt1 increases the firing rate of noradrenergic LC neurons and induces expression of *c-fos* in these cells [69, 238] via depolarization involving both an augmented nonselective cation conductance and an inhibited potassium conductance [239]. Partial ablation of the

LC neurons via Hcrt2 targeting of the neurotoxin saporin (which kills LC neurons inefficiently due to the low affinity of Hcrt2 for Hcrtr1, the sole receptor in the LC) increased both sleep and limb movements during REM without eliciting cataplexy, consistent with the notion (see below on histaminergic systems) that Hcrt excitation of the LC during waking periods stimulates arousal and muscle tone and that Hcrt silence during REM serves to repress muscle activity controlled by the brain stem [240].

Hcrt terminals are also found on GABA neurons in the peri-LC [53, 241]. GABA inhibits LC neurons [242], suggesting that Hcrt has both direct excitatory effects on LC noradrenergic neurons and inhibitory effects on these same neurons via a feedforward circuit involving local GABA interneurons. Hcrt neurons are responsive to noradrenaline, thus providing a feedback loop between LC and Hcrt neurons. Whether the noradrenaline effect is excitatory, inhibitory, or state dependent is presently a matter of debate [58, 243, 244].

4.12.3 Serotonergic Systems

Serotonin neurons of the dorsal raphe in the brain stem are part of modulatory ascending and descending pathways that gate sleep–wake states [245]. These neurons receive input from Hcrt fibers which form synapses on them [246]. These serotonergic neurons express both Hcrtr1 and Hcrtr2 [247] and are equally excitable by Hcrt1 and Hcrt2, suggesting that signaling occurs through both receptors [248]. These neurons are sensitive to Hcrt excitation, which is of slow onset and long duration, during slow-wave or REM sleep, but not during wakefulness during which these neurons are already maximally active [249]. Hypocretins increase the activity of serotonin cells in the dorsal raphe via a PKC-mediated influx of Ca^{2+} through L-type calcium channels [70, 71, 250].

Studies utilizing hypothalamic slices from transgenic mice expressing green fluorescent protein exclusively in Hcrt neurons demonstrate that these neurons are hyperpolarized by serotonin via a mechanism involving an inwardly rectifying potassium channel and that the hyperpolarization is blocked in the presence of the 5-HT_{1A}-selective antagonist WAY100635 [43]. ICV administration of WAY100635 to mice in the latter part of night increased locomotor activity, but this effect was not observed in mice in which Hcrt neurons were ablated [43]. Although direct reciprocal inputs from dorsal raphe serotonergic neurons to Hcrt neurons in the LH have yet to be demonstrated, the above findings are consistent with activity-dependent increase in serotonin in the LH contributing to waning Hcrt neuronal activity as the sleep period approaches.

4.12.4 Histaminergic Systems

Histamine neurons are wake-active neurons located exclusively in the TMN of the hypothalamus. They project to the hypothalamus, basal forebrain, thalamus, cortex, and brain stem. TMN neurons express Hcrtr2 and receive inputs from Hcrt-containing axons. Both Hcrt1 and Hcrt2 depolarize histaminergic TMN neurons by activation of an electrogenic sodium–calcium exchanger and a Ca^{2+} current associated with a small decrease in input resistance and increases in spontaneous firing [77]. Knockout mice deficient in histamine receptor 1 are impervious to

hypocretin administration, suggesting that at least some of the effects of the Hcrt are caused by release of histamine and activation of postsynaptic H_1 receptors [78, 251, 252]. The concentrations of the mRNAs for histamine receptors decrease with age in the mouse brain, as does *Hcrtr2* mRNA, possibly accounting the diminishment of alertness and quality of sleep associated with aging [253].

One of the features of narcolepsy is cataplexy, which is a sudden loss of skeletal muscle tone, often triggered by emotions or laughter. During cataplectic episodes, although narcoleptic individuals enter a REM-like state of muscle atonia, they are awake, aware of their environment, and otherwise conscious. Thus, vigilance and the control of muscle tone are dissociated in this pathological state. Studies in *Hcrtr2*-deficient narcoleptic dogs [254] showed that histamine neurons, in contrast to noradrenergic and serotonergic REM-off cell groups, are active during cataplexy. Activity of histamine neurons is thus linked to the maintenance of waking, whereas that of noradrenergic and serotonergic neurons is tightly coupled to the maintenance of muscle tone in waking and its loss in REM sleep and cataplexy.

4.12.5 Dopaminergic Systems

The dopaminergic neurons of the VTA and ventral periaqueductal gray do not change their activity greatly throughout the sleep–wake cycle. Dopaminergic and GABAergic neurons in the VTA receive inputs from Hcrt neurons of the LH [170]. The hypocretins excite these neurons via Ca^{2+} -, PLC-, and PKC-dependent pathways, promoting arousal [89]. Interestingly, administration of dopamine agonists to the VTA of *Hcrtr2*-deficient dogs aggravated cataplexy, suggesting that this structure contributes to inhibition of muscle tone during REM [255].

4.12.6 Cholinergic Systems

The hypocretin neurons project to cholinergic brain stem REM-on neurons, including those in the LDT and the pontine reticular formation whose projections contribute to cholinergic tone in the forebrain. This tone is elevated during both wakefulness and REM leading to desynchronization of the EEG. Cholinergic tone is low in slow-wave sleep and during which acetylcholine activity is further inhibited by the sleep-promoting peptide of forebrain interneurons, cortistatin [256], contributing to the slow-wave synchrony of the EEG. In contrast *Hcrt1* enhances cortical release of acetylcholine [257]. Local injection of *Hcrt1* into the LDT of freely moving cats increases wakefulness and decreases the number of REM episodes but does not influence episode length [220], suggesting that the hypocretin system influences the gate (or switch) to REM by reducing the firing rates of the brain stem REM-on cells but does not itself operate during REM. This and the fact that deficiencies in the hypocretin system lead to increases in REM episodes make it more likely that action at REM-on structures by hypocretin occurs only during waking periods [258]. The role of hypocretin in regulating the REM gate is a complex one in that, paradoxically, the REM-on structures receive both indirect hypocretin-initiated inhibitory signals from REM-off cells and direct projections from the hypocretin neurons themselves and therefore must decide on how to respond to this push–pull pressure in different scenarios.

The midline and intralaminar thalamic neurons coordinate activity levels broadly across cerebral cortex and support attention and awareness. *Hcrt1* and *Hcrt2*

selectively depolarize midline–intralaminar thalamic neurons and not sensory thalamic neurons [83]. Midline–intralaminar nuclei express Hcrtr2 [60, 61, 259]. The hypocretins excite arousal-related cholinergic neurons in the basal forebrain by suppressing the activity of an inwardly rectifying potassium channel [80, 260].

Hcrt neurons project directly to the cerebral cortex in addition to projecting onto subcortical relay neurons. In the cortex, Hcrt fibers are distributed through all layers, although most densely in the deeper layers. In layers 1–6a, hypocretins have no direct effect upon cortical neurons, although there are indirect effects through presynaptic terminals of inputs [81]. Hcrt has a direct postsynaptic depolarizing action upon cortical neurons located exclusively in layer 6b that is mediated by Hcrtr2. Layer 6b neurons project diffusely to layer 1 of surrounding cortical areas [261], allowing cortical activation to propagate widely.

Hcrt fibers form synapses on cholinergic neurons in the medial septum–diagonal band of Broca [79]. Both Hcrt1 and Hcrt2 activate these neurons via a mechanism that involves both an inwardly rectifying potassium channel and a probable sodium–calcium exchanger [79]. These neurons, which express Hcrtr2 and show axon degeneration in Hcrtr2-deficient dogs, project to the hippocampus, where they influence local theta rhythm and its associated memory functionality.

4.13 HYPOCRETINS INTEGRATE AROUSAL, FEEDING BEHAVIOR, AND MOTIVATION

Hypocretin peptides excite LC and dorsal raphe neurons to elevate muscle tone and TMN neurons to promote wakefulness. These components of the ascending reticular activating system, and the Hcrt neurons themselves, project to and stimulate thalamic and basal forebrain neurons, and all of these groups contribute to the depolarization of the cerebral cortex, including layer 6b neurons (Fig. 4.2). Arousal-related signaling occurs through both Hcrtr1 and Hcrtr2. The involvement of both receptors is consistent with the more severe phenotype of the double receptor knockout mice compared to either single receptor knockout [196]. It is also consistent with the observation that sporadic cases of canine narcolepsy associated with lower or undetectable CSF hypocretin are more severe than are cases with the Hcrtr2 deficiencies alone [262]. Nevertheless, Hcrtr2 plays the more prominent role in raising arousal levels.

These peptides also have diverse effects on brain reward and autonomic systems related to stress that serve to increase motivated behaviors, among those feeding. The relation to feeding is a complex one. Acute administration of Hcrt to sleeping rats increases food consumption. However, patients with narcolepsy have chronically low concentrations of the hypocretins but have an increased likelihood of being obese despite reduced daily calorie intake [263–265], and in children, plasma Hcrt concentrations are negatively correlated with body mass index [266]. Similarly, although hypocretin knockout mice are hypophagic, they do not have lower weights than the unaffected controls. Mice depleted of hypocretin neurons fail to show increased alertness in response to fasting, suggesting that the hypocretinergeric system senses changes in metabolic signals and drives the circuitry involved in arousal. Rather than considering the hypocretins to be orexigenic, they appear to serve as a counterregulatory response to obesity.

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5

PROKINETICINS: NEW PAIR OF REGULATORY PEPTIDES

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5.1	Introduction	163
5.2	Receptors for Prokineticins	164
5.3	Structural Determinants Required for Functional Activities of Prokineticins	164
5.4	Prokineticin as Regulator of Smooth Muscle Contractility	166
5.5	Prokineticins and Pain Perception	166
5.6	Regulatory Function in Circadian Clock Output	167
5.7	Functions in Ingestive Behavior	168
5.8	Functions in Angiogenesis and Reproduction System	169
5.9	Function in Hematopoiesis	170
5.10	Function in Neurodevelopment	171
5.11	Perspectives	172
	References	172

5.1 INTRODUCTION

Snake venom and skin secretion from frogs have been rich sources for isolating biologically active regulatory peptides. Prokineticins (PKs) are no exceptions. The history of PKs can be traced back to more than 20 years ago, when Joubert and Strydom purified a 77-amino-acid peptide from black mamba venom with unknown biological functions [1]. This peptide was named MIT1, and the first biological function associated with this family of peptides was its ability to stimulate the contraction of gastrointestinal smooth muscle [2]. The real expansion of the field started when Kreil's group purified and sequenced a frog protein named Bv8 [3]. This frog protein was shown to be highly similar to the snake protein and possess the same potent contractile activity on gastrointestinal smooth muscles [3]. The mammalian homologs for Bv8 and MIT1, with consensus name of PKs, were subsequently identified by three groups [4–6]. Over the last few years, a spectrum of biological functions has been assigned to this new family of regulatory peptides (Fig. 5.1).

5.2 RECEPTORS FOR PROKINETICINS

Three groups independently identified two closely related G-protein-coupled receptors for PKs, prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2) [7–9]. These receptors are unusually conserved with over 80% amino acid identity between them. Recombinant PK1 and PK2 have been shown to activate both receptors at nanomolar concentrations when exogenously expressed in Chinese hamster ovary (CHO) cells. In PKR-transfected cell lines, neurons, and specific endothelial cells, activation of prokineticin receptors stimulates calcium mobilization, phosphoinositol turnover, mitogen-activated protein kinase (MAPK), and Akt kinase pathways [7–10]. While the stimulation of calcium mobilization upon receptor activation is dependent on G_q , activation of the MAPK pathway is pertussis toxin sensitive, suggesting that PKR may also couple to other G proteins such as G_i . Activation of the G_i signaling may also be related to the function of PK in cell proliferation, migration, and survival.

Reverse transcriptase polymer chain reaction (RT-PCR) indicates that PKR1 is widely distributed in the periphery, including the stomach, small intestines, colon, spleen, pancreas, and peripheral leukocytes [7–9]. Although both receptors are expressed in the gastrointestinal (GI) system, PKR1 is more abundantly expressed in the stomach and intestine, suggesting that it may be the major receptor mediating the actions of PKs in GI motility. Whereas PKR1 is more widely expressed in the periphery, *in situ* analysis indicates that PKR2 is the more dominantly expressed receptor in the adult brain. PKR2 messenger RNA (mRNA) can be detected throughout the adult brain, including areas in the hypothalamus and its primary output targets, as well as the olfactory ventricular regions and the limbic system [11, 12]. RT-PCR also indicates that PKR are expressed in various endocrine tissues, including thyroid, pituitary, adrenal gland, testis, and ovary [7–9]. The presence of both receptors in several endocrine and nonendocrine tissues and their activation of the MAPK pathway may be related to the function of PKs in angiogenesis, cell proliferation, migration, and survival [6]. Furthermore, Negri et al. [13] revealed the presence of both PKRs in the dorsal root ganglion and the spinal cord, and thus the effects of Bv8 on nociceptive threshold may result from the activation of PKRs in these sensory neurons.

5.3 STRUCTURAL DETERMINANTS REQUIRED FOR FUNCTIONAL ACTIVITIES OF PROKINETICINS

The mammalian prokineticins (PK1 and PK2) have about 45% amino acid identity between them and also to their nonmammalian counterparts (MIT1 and Bv8). Sequence analysis indicates that both the human and mouse PKs contain three exons with distinct N-terminal and C-terminal domains [14]. The first exon encodes a signal peptide and the first five amino acids in the N-terminus, and exons 2 and 3 encode the cysteine-rich domains. Interestingly, PKs and their amphibian homologs exhibit certain conserved characteristics in their amino acid sequences. This includes the first six amino acids in the N-terminal domain (AVITGA) and the 10 cysteine residues with identical positions, which are predicted to form five pairs of disulfide bonds [5]. These conserved characteristics suggest the potential functional importance of

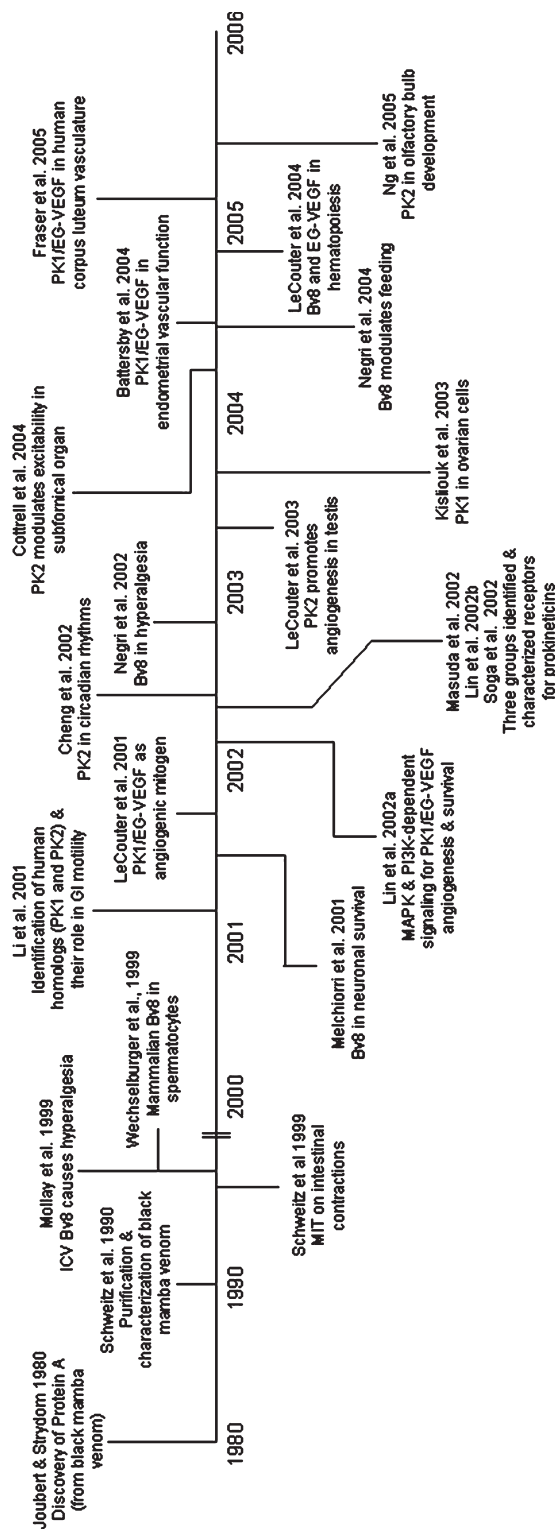


Figure 5.1 Timeline of discovery of biological functions for prokineticins/Bv8/EG-VEGF.

these residues. To delineate the structural elements that are essential for the bioactivities of PKs, we have investigated the structural–functional relationship of PKs by generating recombinant proteins that contain substitutions, deletions, or insertions of amino acid in the N-terminal or the C-terminal [14]. These mutant proteins were then tested for their agonist activities by measuring their ability to mobilize intracellular calcium and/or induce ileum contractions. Substitution or addition of any of the six amino acid residues in the N-terminus rendered the PKs inactive, indicating the importance of these N-terminal residues for activating PKRs. Mutation studies have also demonstrated that the conserved cysteine residues are also critical for their bioactivities, although residue changes in the C-terminus are more tolerable. Chimeric proteins with exchanged cysteine-rich domains between PK1 and PK2 remain active and possess functional activities comparable to PK1 or PK2. Intriguingly, two of the N-terminus mutants with either substitution or addition of only one amino acid generated mutant PKs that possess potent antagonist activity. A splice variant of PK2 that contains an extra 21-residue insertion in the C-terminus is also biologically active, although with a much lower potency. A recent study reported that there might be further protease processing of this PK2 variant, which yields another peptide that may specifically activate PKR1 in stimulating adenylate cyclase [15, 15a]. It is likely that PK ligands may be more complicated than originally thought.

5.4 PROKINETICIN AS REGULATOR OF SMOOTH MUSCLE CONTRACTILITY

The motility of the GI tract is regulated by classical neurotransmitters, neuropeptides, and circulating hormones. Schweitz et al. [2, 16] had purified MIT1 from snake venom and demonstrated that it potently stimulated contraction of the guinea pig ileum. A protein of similar size that has greater than 40% identity with MIT1 has also been purified from frog skin secretion [3]. This frog protein is named Bv8 and is also found to cause potent contractions of the GI smooth muscles. In an attempt to search for the mammalian homologs of the frog Bv8 and snake MIT1, we have isolated and characterized two human complementary DNAs (cDNAs) that encode the homologs for snake MIT1 and frog Bv8 [5]. We have produced recombinant human PKs and demonstrated that these mammalian proteins also stimulate contractions of the GI smooth muscles with similar potency. To reflect their potent actions on GI contractility, we have named these proteins “prokineticins.” Whereas it is still unclear whether the effect of PKs on GI smooth muscle cells are pharmacological or physiological, subsequent *in vivo* studies have demonstrated that both PKs stimulate the GI transit (Owyang et al., unpublished study, Zymogenetics patent filing). Although the cell identities that express PKs in GI tissues remain unknown, ligand binding studies had revealed the presence of high-affinity receptors for PKs in the GI smooth muscle cells [5].

5.5 PROKINETICINS AND PAIN PERCEPTION

The first observation that linked PKs to nociception was reported by Mollay et al., who observed that administration of Bv8 into the rat brain induced a marked

hyperalgesic response [3]. Based on this initial finding and other preliminary results which indicated that pituitary and circulating leucocytes express high level of PK2 [7–9, 13], the same group speculated that these proteins might function as inflammatory agents. In a further study, Negri et al. assessed the effects of frog Bv8 on the nociceptive threshold to thermal and mechanical stimuli using various routes of administration [13]. Intravenous, subcutaneous and intrathecal injections of Bv8 all produced intense systemic nociceptive sensitization to mechanical and thermal stimuli applied to tail and paws [13]. Topical delivery of Bv8 also decreased the nociceptive threshold. To elucidate whether the hyperalgesic effects of Bv8 may be mediated through PKRs, they have examined the localization of PKRs in the rat dorsal root ganglia (DRG) and the spinal cord. Both PKR1 and PKR2 mRNAs are expressed in the DRG and the spinal cord [13]. It was also demonstrated that Bv8 can bind to these PKRs on the DRG neurons and the spinal cord with high affinity. Furthermore, functional assays indicated that Bv8 was able to mobilize calcium in cultured rat DRG neurons in a dose-dependent manner [13]. These data suggest that Bv8 or its mammalian homologs sensitize peripheral or central nociceptors via activation of PKRs in the DRG or spinal cord.

5.6 REGULATORY FUNCTION IN CIRCADIAN CLOCK OUTPUT

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the primary mammalian circadian clock that regulates daily rhythms of diverse physiology and behavior, including sleep–wake cycle, blood pressure, heart rate, body temperature, and energy metabolism [17]. While the understanding of the molecular mechanism of the circadian clockwork has emerged in the last decade [17, 18], little is known about the mechanism by which the circadian clock transmits timing information to control physiology and behavior. In an attempt to study the function of PKs in the central nervous system, we have mapped the mRNA distribution of the PKs and their receptors in the mouse brain using *in situ* hybridization analysis. Our initial characterization revealed that PK2 mRNA is highly expressed in the SCN in an oscillating fashion, with high levels during the day and low levels or undetectable at night [11]. Subsequent molecular and behavioral studies demonstrated that PK2 is a candidate output signaling molecule for circadian locomotor rhythms [11]. The PK2 oscillation in the SCN is maintained in animals under normal 12 h light–12 h dark as well as under constant darkness, indicating that this PK2 oscillation is driven by the endogenous circadian clockwork [11]. As Clock and Bmal1 are the positive elements of the clockwork, these transcription factors heterodimerize and bind to CACGTG sequences known as E-box to activate transcription of other clock or clock-controlled genes [17, 18]. Both the human and mouse PK2 promoter contains four E-boxes within the first 2 kb upstream of the transcription start site [11]. *In vitro* reporter gene assay indicated that PK2 transcription is tightly regulated by clockwork gene products through activation of the E-boxes residing in its proximal promoter. These *in vitro* findings were supported by *in vivo* studies which demonstrated that PK2 mRNA expression in the SCN is completely absent or blunted in mutant mice that lack functional clockwork. *In situ* hybridization revealed that the receptor for PK2 is expressed in primary SCN output target areas, further supporting the role of PK2 in circadian rhythms. To elucidate whether PK2 plays a role in

circadian-regulated behavior, we have tested the effects of PK2 on locomotor wheel-running activity, as this is one of the most common ways to study circadian rhythms. Intracerebroventricular delivery of PK2 into the lateral ventricle during subjective night, when endogenous PK2 is low, inhibited the nocturnal wheel-running activity of rats [11]. These results demonstrated that PK2 is a candidate output molecule that transmits circadian behavioral rhythms from the SCN clock.

While the direct genetic analysis of PK2 deficiency on circadian rhythm has yet to be determined, Morton et al. have observed a correlation between increased daytime activity and reduced SCN expression of PK2 molecular rhythm in a transgenic model of Huntington's disease [19]. As PK2 is a SCN output molecule regulating circadian locomotor behavior, this finding suggests that the reduced PK2 rhythm may contribute to the sleep disturbances and abnormal circadian behavior seen in this strain of transgenic mice. Recently, the molecular rhythm of PK2 in the SCN of a diurnal rodent has been reported [20]. Similar to the oscillation pattern observed in mouse, PK2 mRNA in the SCN of *Arvicanthis niloticus* was also rhythmically expressed, with peak levels in the morning, preceding the peaks of *Per1* and *Per2* [20]. Thus, the phase of PK2 expression in the SCN of diurnal rodent is similar to that of nocturnal rodents, consistent with a growing body of evidence suggesting that the key to diurnality lies downstream of the SCN circadian clock.

As we have previously observed that nocturnal light pulses can rapidly induce PK2 expression in the SCN, we have further investigated the possibility that PK2 is under the dual regulation of core clockwork and the light. Using a common model for jet lag, we examined how PK2 responds to abrupt shifts of light–dark cycles (phase delays or phase advances) [21]. We observed that PK2 expression exhibits transients in response to phase advances but entrains rapidly to phase delays. Intriguingly, this differential pattern of PK2 expression during time shifts is consistent with the circadian phenomenon that animals and humans adjust rapidly to time delays but slowly to advances [22, 23]. This study further provided additional supporting evidence for PK2 as an output signaling molecule from the SCN. We have also made another interesting observation that light can directly regulate PK2 in the absence of functional circadian clockwork. Studies with cryptochrome double-knockout mice revealed that a light-regulated low-amplitude PK2 rhythm exists in these mice that lack functional circadian oscillators [21]. These studies suggest that PK2 is controlled by dual mechanisms: dominantly by the circadian oscillators but also directly by light. Evolutionarily, this could indicate that PK2 may evolve before the circadian timing system becomes internalized in the brain.

5.7 FUNCTIONS IN INGESTIVE BEHAVIOR

Daily ingestive behaviors such as feeding and drinking are examples of circadian-regulated processes, as rodents consume over 80% of food during the dark period [24]. Since PK2 has been shown to be one of the first circadian output molecules that regulate circadian locomotor behavior, the expression patterns of PK2 also suggest that PK2 may play a role in feeding and drinking, as PK2 mRNA is expressed in areas such as the arcuate nucleus, paraventricular hypothalamic nucleus, and subfornical organ, which are primary regions that regulate ingestive behavior [11, 24, 25]. Thus, it is possible that PK2 could also modulate other circadian behavior,

such as feeding and drinking. To test this hypothesis, Negri et al. examined the effects of Bv8 on spontaneous food and water intake and demonstrated that Bv8 and PK2 modulate these ingestive behaviors in rats [26]. They showed that intracerebroventricular delivery of Bv8 suppressed diurnal and nocturnal feeding as well as deprivation-induced and NPY-stimulated feeding [26]. Bv8 injected into the lateral ventricle also stimulated drinking during daytime. When Bv8 is microinjected into the arcuate nucleus, Bv8 suppressed feeding in normal fed and fasted rats without affecting drinking. Interestingly, microinjection of Bv8 into the subfornical organ, an area in the brain that is known for regulation of thirst [25], stimulated drinking. It has recently been shown that PK2 activates and depolarizes the dissociated neurons of the subfornical organ [27]. Similar to the actions of Bv8, preliminary results showed that the mammalian recombinant PK2 also produced potent anorexigenic response in rats [26], our unpublished observations). Although the current target and pathway of Bv8/PK2 in regulating these ingestive behaviors are still unclear, it is possible that this effect may be related to the role of PK2 as a circadian output molecule, as the SCN is known to regulate diverse physiological processes, including feeding and drinking [24]. These findings indicate that PK2 may be a regulatory molecule for ingestive behavior and further expanded the versatile function of prokineticins in physiology and behavior.

5.8 FUNCTIONS IN ANGIOGENESIS AND REPRODUCTION SYSTEM

During the last decade, significant progress has been made in the identification of molecules that regulate vascular growth, a crucial process for many diverse biological processes, including embryonic development, reproductive functions, tumorigenesis, and wound healing. In particular, vascular endothelial growth factor (VEGF) and the Tie2 ligands (angiopoietins) have been shown to play critical roles in the proliferation of endothelial cells and the assembly of blood vessel wall [28]. However, these molecules are widely expressed and show little tissue specificity in their angiogenic properties and thus cannot account for the tissue-specific angiogenesis. When screening a library of human secreted molecules for their ability to induce proliferation in primary bovine adrenal cortex-derived capillary endothelial [angiotensin-converting enzyme (ACE)] cells, LeCouter et al identified one molecule that was capable of inducing strong mitogenic response [6]. Due to its tissue-specific angiogenic nature, this molecule is named endocrine gland-derived (EG) VEGF. Delivery of EG-VEGF in ovary elicited potent angiogenesis and cyst formation, and this effect is absent when delivered to cornea or skeletal muscles. Similar to VEGF, EG-VEGF also has a HIF-1 binding site, and its expression is induced by hypoxia. Sequence analysis revealed that EG-VEGF is the same molecule as PK1 [5, 6].

Following the initial finding of PK1 as the first example of a tissue-specific angiogenic factor, LeCouter et al. also pursued whether a PK1-related molecule, PK2, also plays a role in angiogenesis. PK2 has been shown to induce proliferation, survival, and migration of ACE cells, and its expression is also hypoxia inducible [29]. Interestingly, PK2 is highly expressed in the testis, and adenoviral delivery of PK2 into mouse testis resulted in a potent angiogenic response. This angiogenic effect is likely to be mediated through PKR, as both PKRs are expressed in the vascular endothelial cells of the testis [29]. The potent angiogenic effect of PKs suggests their

role in regulating the proliferation of blood vessels in reproductive organs. In the following years, several groups have further examined the role of PKs in reproductive organs, as the process of angiogenesis is crucial for reproductive functions [30–32]. Battersby et al. [31] demonstrated that both PK receptors were localized in the glandular epithelial, stromal, and endothelial cells in the human endometrium. Quantitative PCR revealed that PK1 expression, but not PK2, was elevated during the secretory phase of the menstrual cycle, and this PK1 elevation is induced by treatment with progesterone [31]. Interestingly, two putative progesterone receptor binding sites are found in the promoter of PK1, suggesting that the increase in PK1 expression may be caused by the direct action of progesterone on PK1 transcription [31]. Another group examined the regulation of VEGF and PK1 in human ovarian granulosa cells and demonstrated that both of these angiogenic factors are expressed in these cells but their expression is inversely regulated [30]. It is interesting to note that in these cells PK1 expression is stimulated by forskolin but downregulated by chemical hypoxia and thrombin [30]. This opposite effect may be due to the cell type that is expressing PK1. Frazer et al. [32] recently demonstrated that PK1 is localized in the human corpus luteum and its expression is increased throughout the luteal phase. Unlike the increase of PK1 in the human endometrium, the expression of PK1 in human corpus luteum is increased by treatment with human chorionic gonadotropin but not influenced by progesterone [32]. Together these studies indicate that PKs have a physiological role in reproductive function by regulating endothelial or other cell types.

Several groups have also studied the expression of PK1 in ovarian diseases such as polycystic ovary syndrome (PCOS) and ovarian tumors [33, 34]. A prominent feature of PCOS is an increased angiogenesis, leading to hyperplasia and hypervascularity of the ovarian theca interna and stroma. As PK1 has a selective mitogenic effect for the endothelium of steroidogenic glands [6], Ferrara et al. [33] examined the expression of PK1 in human PCOS and normal ovary specimens. In PCOS ovaries, strong expression of PK1 mRNA was detected in theca interna and stroma, whereas VEGF mRNA was mainly expressed in the granulosa cell layer [33]. This complementary expression pattern is an amplification of the pattern seen in normal ovaries. These findings indicate that PK1 and VEGF may play a complementary function for ovarian angiogenesis and possibly cyst formation. Another group focused on examining the expression of PK1 in different stages of ovarian tumor samples [34]. Although PK1 mRNA was detected in all ovarian carcinomas, including benign, low malignant potential neoplasms, or stage I ovarian cancer, no significant differences were identified between these tumor types. It is interesting to note that PK1 mRNA level was also lower in these tumor samples when compared to normal premenopausal ovaries. During the later stages of these ovarian carcinomas, a significant further decrease of PK1 mRNA was observed [34]. In contrast to VEGF, PK1 mRNA levels did not correlate with clinical outcome in advanced ovarian carcinoma. These results suggest that PK1 may only play a marginal role in promoting angiogenesis in advanced ovarian carcinoma.

5.9 FUNCTION IN HEMATOPOIESIS

Since PK1 and PK2, as well as their receptors, are known to express in bone marrow and other hematopoietic organs [5], LeCouter et al. [35] investigated a possible role of

PK1 and PK2 in hematopoiesis. Detailed expression analyses indicated that both PKR1 and PKR2 are expressed in hematopoietic stem cells and specific mature blood cells, including lymphocytes. Interestingly, PK2 is also highly expressed in infiltrating cells at sites of inflammation, predominantly in neutrophils, and the expression of PK2 and both PKRs are induced in monocytes upon exposure to the endotoxin lipopolysaccharide (LPS) [35]. Le Couter et al. [35] demonstrated that PK2 can induce monocyte migration and this migratory signaling is pertussis toxin sensitive, suggesting that G_i may be the primary G protein mediating this effect. Furthermore, PK2 also increases the number of colony-forming units granulocytic and monocytic in cultures of the human or mouse hematopoietic stem cells. Systemic injection of PK2 also increases the number of leukocyte, neutrophil, and monocyte counts. In addition to the proliferative effects of PK2 on these immune cells, PK2 also promotes their survival, as adenoviral delivery of PK2 increased the survival of hematopoietic cells in 5-fluorouracil-induced injury. This is consistent with the previous finding that Bv8 supports the survival of cortical and cerebellar granule cells in cultures against apoptosis and excitotoxic death [36]. Together, these studies suggest that PK2 may function as a monocyte chemoattractant and a hematopoietic cytokine that promotes survival and modulates the innate and adaptive immune systems.

5.10 FUNCTION IN NEURODEVELOPMENT

In mammals, neurogenesis occurs mainly during embryonic to early postnatal stages. However, there exists persistent neurogenesis in certain regions of adult mammalian brains, including the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus [37, 38]. New neurons are continuously generated in the OB from progenitor cells that reside in the subventricular zone (SVZ) of the lateral ventricle, where they proliferate and migrate through the rostral migratory stream (RMS) to the OB to form granular and periglomerular cells [39–41]. Although this SVZ–OB pathway and the timing of neurogenesis have been well documented, the mechanism of this guided process is still not well understood. We have recently demonstrated that PK2 functions as a chemoattractant for SVZ-derived neuronal progenitors and is essential for the normal development of OB architecture [12]. In situ hybridization revealed that the expression of PK2 and its receptor complement each other in the OB, with PK2 expressed in the mature granular and periglomerular layers of the OB whereas its receptors (PKR1 and PKR2) are expressed in the immature ependyma and subependymal layers of the olfactory ventricle (OV). In vitro and in vivo studies demonstrated that PK2 serves as a chemoattractant for the SVZ-derived neuronal precursors. Migration assays using Transwell assay system and collagen gel matrices indicated that PK2 can stimulate migration of neuronal progenitors from the RMS in both adult and postnatal rats, and this migration is directional and can be inhibited by the PK antagonist A1MPK1 [12]. It is also likely that PK2 may act as a detachment signal for the chained migrating neuronal precursors, as addition of PK2 in SVZ explant cultures increases the number of cells migrating out of the explants and disperses the cells from migrating chains to individual cells. The critical role of PK2 in OB development was demonstrated by genetic studies. PK2 deficiency in mice resulted in abnormal development of OB, including dramatic reduction in OB size and the loss of normal OB layer architecture [12]. As PKs have been previously

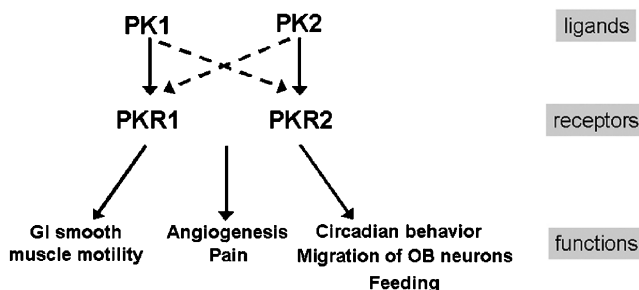


Figure 5.2 Known biological functions of prokineticins. Prokineticins PK1 and PK2 are a pair of cysteine-rich secreted molecules that exert their functions via activation of two G-protein-coupled receptors (PKR1 and PKR2). Activation of prokineticin receptors leads to multiple signaling pathways, including mobilization of intracellular calcium, phosphoinositol turnover, and mitogen-activated protein kinase pathways. Prokineticins have been shown as versatile molecules regulating multiple biological processes, with some selective pairing of ligand and receptor. In particular, prokineticins stimulate gastrointestinal smooth muscle motility and induce hyperalgesia. Prokineticins have also been shown to function as angiogenic mitogens that act selectively on endothelial cells from endocrine glands or reproductive organs. Furthermore, PK2 has been shown as a candidate output signaling molecule that regulates circadian behavior, including locomotor activity and feeding behavior. More recently, PK2 has been shown as a critical chemotactic molecule that is essential for the migration of OB neurons.

demonstrated as angiogenic factors, our findings of PK2's role in neurogenesis support that signaling commonality between angiogenesis and neurogenesis.

5.11 PERSPECTIVES

Over the last few years, PKs have evolved as a pair of signaling peptides that regulates diverse functions ranging from GI motility, angiogenesis, and hematopoiesis to circadian clock output and ingestive behavior (Fig. 5.2). It is interesting to note that these diverse functions may be classified into two general categories: cell excitability and cell motility. It appears that the regulatory function of PKs in GI smooth muscle, circadian clock output, and ingestive behaviors may be related to the activation of PKRs in the target cells, which results in enhanced cell excitability, whereas the function of PKs in angiogenesis, hematopoiesis, and neurodevelopment seems to be more closely linked to the effect of PKs in modulating cell motility. Recently, in collaboration with the Ferguson group, we have directly demonstrated that PK2 activates and depolarizes the neurons of the subfornical organ and the paraventricular hypothalamic nucleus [27, 42]. Another theme that is emerging from these studies is that the transcripts of PKs are dynamically regulated. Without a doubt, the current known functions of the PK system are likely to be just the tip of an iceberg.

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6

SEDATIVES AND HYPNOTICS

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6.1	Introduction	177
6.2	GABA _A Receptors	178
6.2.1	Structure	178
6.2.2	Pharmacology	180
6.2.2.1	Benzodiazepine Binding Site	180
6.2.2.2	Barbiturate Binding Site	181
6.3	Sites of Action in Brain for Hypnotics/Sedatives	181
6.4	The Development of Safer and More Selective Hypnotics	184
6.4.1	Barbiturates	184
6.4.2	Barbiturate-Like Drugs	187
6.4.3	Benzodiazepines	187
6.4.4	Non-GABA _A Receptor Agents	188
6.4.5	Potential New Therapies	188
6.5	Genetically Modified Mice as Tools to Understand Function of Hypnotics/ Sedatives	190
6.6	Future Directions	192
	References	192

6.1 INTRODUCTION

Sedatives and hypnotics are general terms to represent a class of drugs that induce a general depression of central nervous system (CNS) function. As will be discussed below, there are a number of different types of sedatives/hypnotics. They have evolved over the years from what could be defined as broad-spectrum CNS depressants such as the barbiturates through the much more specific agents (in terms of their molecular targets) [1]. The vast majority of sedatives/hypnotics have their clinical effect by modulating the γ -aminobutyric acid type A (GABA_A) receptor system in the brain. They are exemplified by the ubiquitous benzodiazepines, such as diazepam

(Valium). As such, GABA_A receptor modulators will be the primary focus of this chapter.

The clinical need for drugs classed as hypnotics/sedatives is clear. Use of drugs such as the benzodiazepines as sedatives in clinical practice is widespread [2]. They are used in surgery preoperatively, and indeed in minor operative procedures, to induce myorelaxation and sedation as well as in the emergency room as acute relaxants. Beyond these acute uses, as would be expected, there is widespread and growing use as treatment for insomnia [3, 4]. Insomnia can be defined as difficulty in sleeping or disturbed sleep patterns leaving the perception of insufficient sleep. Insomnia is divided generally into two main categories: sleep onset insomnia (the inability to fall asleep naturally) and sleep maintenance insomnia (the inability to stay asleep or to resume sleep after waking in the middle of the sleep cycle). Insomnia can be further categorized as acute or chronic. Acute insomnia is self-limiting, lasting a few weeks or months and ending without being treated. Chronic insomnia lasts longer than three months. The prevalence of insomnia is unknown but may well affect at least half the adult population [5]. Because insomnia can be a symptom of an underlying illness, stress, trauma, and so on, its incidence varies with regard to age, sex, and severity of the predisposing condition.

In this chapter we will describe what we currently know about the GABA_A receptor, the site of action of the vast majority of currently available sedatives/hypnotics. We will then describe what is known about the neural substrates which are modulated by sedatives/hypnotics to give their clinical effects. Finally we will describe the range of sedative/hypnotic drugs, from barbiturates through to non-selective benzodiazepines, through to subtype selective benzodiazepines and ending with gaboxadol, a selective extrasynaptic GABA_A receptor agonist.

6.2 GABA_A RECEPTORS

6.2.1 Structure

Inhibitory neurotransmission in the adult mammalian CNS is primarily mediated through the GABAergic system [6]. Neurotransmitter receptors for the neurotransmitter GABA are divided into the ionotropic GABA_A and the metabotropic GABA_B, and it is the former that is the topic of this section. GABA_A receptors mediate the majority of fast inhibitory neurotransmission. After GABA is released from the presynaptic terminals, it rapidly diffuses across the synapse and binds to the receptor. Binding of GABA to the two cognate sites on the extracellular surface of the receptor leads to the very rapid (millisecond) opening of the integral ion channel which gates chloride ions [7]. These pass through the ion channel into the postsynaptic cell, causing a hyperpolarization. The flow of ions is terminated by dissociation of the GABA and inactivation of the ion channel. GABA_A receptors are members of the cysteine loop ligand gated ion channel superfamily exemplified by the nicotinic acetylcholine receptor from *Torpedo* electroplax. They are pentameric hetero-oligomers assembled like staves of a barrel around a central ion channel [8]. The topology of the subunits is such that each has a large extracellular domain, four transmembrane domains (M₁–M₄), and a so-called large intracellular loop between M₃ and M₄ [9]. The latter contains several phosphorylation sites which are known to

regulate receptor function and turnover [10]. The pore of the ion channel itself is formed from the M₂ domains of the five subunits. To date, 19 GABA_A receptor subunits (α_1 – α_6 , β_1 – β_3 , γ_1 – γ_3 , δ , ϵ , θ , π , ρ_1 – ρ_3) have been identified [11]. Diversity is further increased by the existence of alternatively spliced versions for a number of these subunits [12]. Each of the subunits has its own unique pattern of expression throughout the CNS, and indeed specificity exists at the level of cell type and probably at the level of the synapse [13]. Clearly the number of potential permutations of assembly of these various subunits into pentameric hetero-oligomers is enormous. Indeed, the question of which subunit combinations actually exist in the mammalian CNS has been a significant challenge and has yet to be completely clarified. It is clear that as techniques have evolved, the more combinations have been identified. It is an interesting point of conjecture as to when the approaches become so refined that they identify subunit assemblies that are errors in the cell's assembly processes. It seems reasonably clear that the composition of receptor subtypes is determined by the regional and cellular distribution of subunits and also by certain rules that govern assembly. Quantitatively, the majority of GABA_A receptors contain a γ_2 subunit. It is almost universally agreed on that the most abundant receptor subtype has the composition $\alpha_1\beta_2\gamma_2$ [14]. The other most abundant receptor subtypes are composed of $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, $\alpha_5\beta_n\gamma_2$, and $\alpha_6\beta_n\gamma_2$. There is also evidence that some GABA_A receptor subtypes contain two different α subunits [15, 16]. The subtype $\alpha_6\beta_n\gamma_2$ is almost exclusively expressed in cerebellar granule cells [17]. The subtype $\alpha_4\beta\gamma_2$ is a relatively minor population [14]. The δ subunit is thought to be largely assembled with α_4 or α_6 (and a β subunit) [18, 19]. While the latter are restricted to cerebellar granule cells, the former are expressed more widely in hippocampal, cortical and, thalamic regions. The receptor subtype $\alpha_4\beta\delta$ is increasingly interesting and is discussed further below in the context of gaboxadol. Far less is known about GABA_A receptors containing γ_1 , γ_3 , ϵ , and π other than these subunits likely substitute for the γ_2 subunit in the receptor complex [20]. Receptors containing ϵ and π certainly are of low abundance with very restricted expression in the CNS [21, 22]. The θ subunit may assemble with α , β , γ subunits but remains an enigma [23]. The ρ subunits have been classified as "GABA_C" receptors, but there is now data to suggest that they can in fact coassemble with α and γ subunits [24].

Protein biochemistry and molecular approaches have been used to further define the structure of the pentameric complex. For the most abundant " α – β – γ " arrangement it is now reasonably well agreed that the stoichiometry is $\alpha(2)\beta(2)\gamma(1)$ in the order α – β – α – β – γ [25]. The GABA binding site is constituted by amino acid residues from both the α and β subunits, while as discussed below the benzodiazepine binding site is constituted by amino acids from the α and γ subunits [26, 27]. Given the above subunit arrangement, this explains why there are two GABA binding sites and one benzodiazepine binding site. An important breakthrough in the understanding of the structure of GABA_A came from the elucidation of the X-ray crystallographic structure of the snail acetylcholine binding protein [28]. In essence this protein is an assembly of the extracellular domains of a cysteine loop ligand gated ion channel and as such was ideal for structural studies. The elucidation of the structure of this molecule provided a molecular template upon which other members of the ion channel family, such as the GABA_A receptor, could be mapped, allowing at the very least the generation of models and predictions for experimental investigation.

An exciting concept that is gaining increased attention is that of extrasynaptic receptors [29]. Neurotransmitter receptors are traditionally thought of as being located directly at the synapse, mediating so-called phasic neurotransmission. Evidence is now gathering to suggest that some neurotransmitter receptors are located in the membrane outside the synapse (are *extrasynaptic*) where they respond to “ambient” concentrations of neurotransmitter primarily as a result of diffusion out of the synapse (so-called tonic neurotransmission) [30]. These receptors would influence the overall level of excitability of that neuron and thereby have a potentially profound effect upon its function [31]. GABA_A receptor $\alpha_5\beta_n\gamma_2$ and also GABA_A δ -containing receptors ($\alpha_4\beta_n\delta$ and $\alpha_6\beta_n\delta$) are thought to be predominantly extrasynaptic [32–34]. This will become important when considering the mode of action of gaboxadol (see below). Certainly the functional properties of δ -containing receptors (high affinity for GABA, and therefore activated by the low concentrations of the neurotransmitter that would be found outside the synapse, and a slower desensitization rate) are consistent with such a role.

6.2.2 Pharmacology

The GABA_A receptor has an extremely rich pharmacology. This is summarized in Figure 6.1. The activity of GABA is allosterically modulated by a significant number of different classes of agents acting through what are believed to be independent binding sites. Since the topic of this chapter is hypnotics and sedatives, we will focus our discussion on the benzodiazepine and barbiturates.

6.2.2.1 Benzodiazepine Binding Site. Before the cloning of complementary DNA (cDNA) encoding receptor subunits revealed the diversity of the gene family, GABA_A receptors were defined by their benzodiazepine pharmacology (the so-called

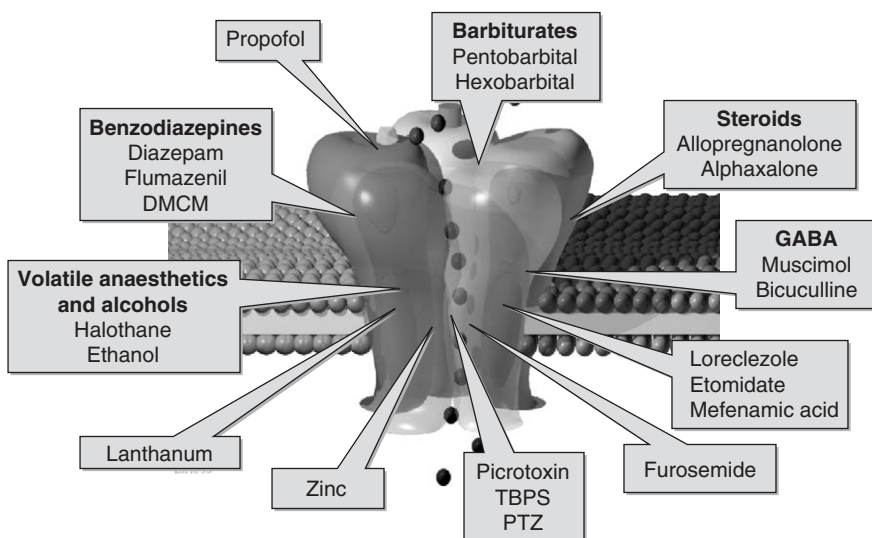


Figure 6.1 Schematic of GABA_A receptor illustrating sites for allosteric modulation. (See color insert.)

BZ1 and BZ2 binding sites). The subsequent investigations into the diversity and structure of GABA_A receptors have enabled a significant insight into their benzodiazepine pharmacology. Only GABA_A receptors containing a γ_2 or γ_3 subunit have a high-affinity benzodiazepine binding site, and since the majority of GABA_A receptors contain a γ_2 subunit, it is likely these are the receptors through which the pharmacological effects of benzodiazepines are mediated [35]. The type of α subunit present in the receptor determines the benzodiazepine pharmacology of that receptor subtype [36], with the β subunit having relatively little influence [37]. As discussed above, the arrangement of the subunits in the receptor complex dictates that the α and γ_2 subunits are neighbors, and indeed the benzodiazepine binding site is constituted by determinants from both the α and γ_2 subunits [27]. Site-directed mutagenesis studies, together with photoaffinity labeling experiments using radiolabeled benzodiazepines, have enabled the identification of a number of amino acid residues in both the α and γ_2 subunits that contribute to the benzodiazepine binding site [38]. While these studies have certainly allowed us to identify the individual amino acids that influence benzodiazepine pharmacology, the resolution and interpretation is still some way from allowing us to use such information to guide rationale drug design. However, a step in this direction comes from the elucidation of the structure of the snail acetylcholine binding protein discussed above; the structure of this protein has been used as a scaffold upon which to lay the amino acid sequences of the GABA_A receptor subunits and thereby visualize the putative structure of the benzodiazepine binding site [27]. A number of the amino acid residues identified by site-directed mutagenesis studies did indeed appear to cluster in the vicinity of each other [39].

6.2.2.2 Barbiturate Binding Site. The site, or indeed the subunit(s), on the GABA_A receptor to which barbiturates bind to exert their activity is currently unknown. This lack of understanding is primarily due to the lack of suitable tools (e.g., high-affinity radiolabeled barbiturates) and the lack of significant selectivity between receptor subtypes, which means site-directed mutagenesis studies which we extensively used to map the benzodiazepine site cannot be so easily applied. What is clear is that barbiturates have two modes of action; at low concentrations they allosterically potentiate the activity of GABA at the receptor, while at high concentrations they can directly activate the receptor in the absence of any GABA [40]. The physiological action of barbiturates is discussed further below.

6.3 SITES OF ACTION IN BRAIN FOR HYPNOTICS/SEDATIVES

It was originally thought that, since GABA receptors are responsible for the primary inhibitory mechanism throughout the CNS, potentiation of this system would result in an overall dampening of activity. Increasing inhibition throughout the entire CNS would logically result in reduced activity and hence sedation. With greater understanding of brain function, this idea is now viewed as rather an oversimplification, and research is very much focused on drug interactions within specific neuronal pathways important for sleep and sedation. To date there have been two major networks identified in the brain that control the sleep–wake cycle. Both originate in the brain stem and project up via different routes to the cortex. The first pathway

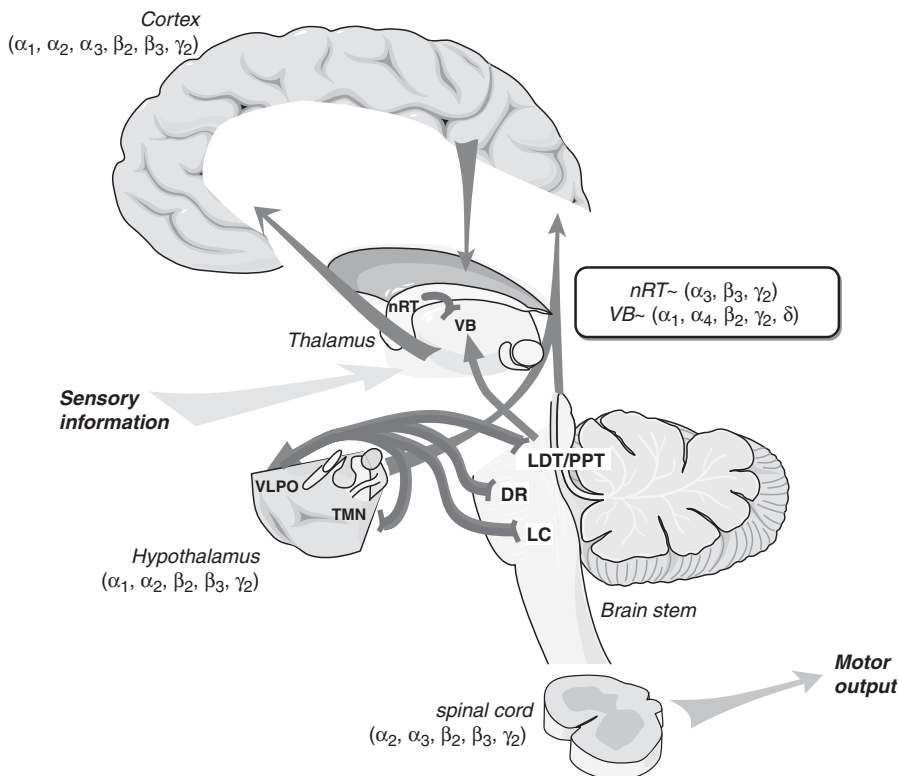


Figure 6.2 Key pathways involved in sleep generation and control. Important brain regions are illustrated with excitatory pathways shown in red and inhibitory pathways in blue. The GABA_A receptor subunits found in each brain region are included highlighting presence of different subtypes. Abbreviations; DR, dorsal raphe; LC, locus ceruleus; LDT/PPT, lateral tegmental nuclei/pedunculopontine nucleus; nRT, nucleus reticularis; TMN, tuberomammillary nucleus; VB, ventrobasal nucleus; VLPO, ventrolateral preoptic nucleus. (See color insert.)

projects through the hypothalamus, while the second projects from the pedunculopontine and lateral tegmental nuclei through the thalamus (Fig. 6.2) [41–44].

Three key nuclei are involved in the hypothalamic pathway; the LC in the brain stem sends a noradrenergic projection to the VLPO nucleus. The VLPO nucleus in turn sends inhibitory GABAergic signals to all the major ascending monoaminergic nuclei, including the TMN, dorsal raphe, and a reciprocal connection back to the LC [45, 46]. VLPO neurons show intense firing activity during sleep, and the increased GABAergic transmission inhibits arousal circuits projecting to the cortex [47, 48]. During arousal, excitatory projections onto the VLPO from the LC, dorsal raphe, and TMN inhibit VLPO activity, in turn reinforcing their own firing and promoting wakefulness [49, 50]. This reciprocal arrangement creates a bistable state of either sleep or waking, with rapid transitions from one state to the other [41]. Using c-Fos as an activity label, increased staining is observed in the VLPO nucleus during sleep; in addition the majority of these c-Fos-labeled cells were also GABAergic [51]. In contrast lesioning the VLPO results in animals that have reduced sleep and increased periods of wakefulness [52]. GABA transmission in this pathway is a critical

component [53], and subcutaneous injection of the GABA antagonist gabazine reduces sedation induced by the systemically administered GABA agonist muscimol as well as the anaesthetics propofol and pentobarbital [54]. A recent study focused on the TMN, which receives GABA inhibition from the VLPO nucleus and sends a histaminergic projection to the cortex. Direct injection of muscimol into the TMN produced sedation, and injection of gabazine reduced the sedative effects of propofol and pentobarbital, suggesting that cortical innervation from this nucleus is important in the control of arousal [54]. Interestingly injection of muscimol into the LC did not induce sedation, suggesting that GABA is not so important in this particular projection; however, injection of the sedative α_2 -adrenergic agonist dexmedetomidine inhibits activity in the LC and promotes VLPO activation [55]. Injection of gabazine into the TMN also reduced dexmedetomidine-induced sedation, suggesting that the adrenergic effects feed directly into the GABA-mediated pathway [55]. One recent paper questions the role of the TMN histaminergic innervation showing that using a saponin–hypocretin conjugate to lesion cells in the TMN results in little direct disruption of spontaneous sleep; however, effects on GABA hypnotics were not studied [56].

The second important pathway for control of sleep is the midbrain to thalamus and thalamocortical connection. The PPT–LDT sends a cholinergic projection to the thalamus and, in contrast to the VLPO nucleus, is inactive during non–rapid-eye-movement (REM) sleep but highly active during wake and also during REM sleep [57]. During wake, sensory information is also directed to the thalamus, which then relays this information on to the cortex, and incoming information can be modulated at this level [58]. The activity of the thalamus is controlled by the surrounding nRt, a layer of cells which projects exclusively into the thalamus via a GABAergic mechanism [59]. There is also an intrathalamic network in which GABA plays a major role in controlling the generation of activity through both synaptic and extrasynaptic mechanisms. Control of the flow of information through the thalamus is crucial for sedation, and it is thought that the GABAergic reticular nucleus can act as a gate, reducing the flow of information from external stimuli into the thalamus [59]. Electroencephalographic (EEG) recording reveals some of the properties of brain electrical activity during sleep and wakefulness and shows characteristic patterns of activity in different sleep stages. At sleep onset “spindle waves” (7–14 Hz frequency) appear which last from 1 to 3 s recurring every 3–10 s. These oscillations originate from network activity between the inhibitory neurons in the reticular nucleus of the thalamus and cortical neurons [60]. Slow-wave sleep or delta oscillations occur during deep sleep and follow spindle activity [61, 62]. These are characteristically large-amplitude, low-frequency waves, and it is believed that spindling followed by delta oscillations result in synchronous pulses of calcium within thalamic and cortical dendrites without producing excessive neuronal firing [58]. These conditions are ideal for promoting gene expression and synaptic plasticity [63, 64] and the possible consolidation of memories, which is believed to occur during sleep [65]. These slow synchronous thalamocortical oscillations are abolished during wake and also during REM sleep. These short bursts of activity are characterized by high-frequency, low-amplitude EEG patterns indistinguishable from those during waking. It is likely that these short bursts of REM represent recall of information acquired during waking which is then reorganized and stored by the highly synchronized events during slow-wave sleep [66, 67].

Clearly GABAergic inhibition plays a major role in both these pathways, and drugs which interfere or modulate this activity will have effects on sleep architecture and wakefulness and consequently the process of memory consolidation. The studies by Nelson et al. [54] highlight specific nuclei in the hypothalamic pathway where GABA modulators such as barbiturates and benzodiazepines will produce sedative effects; however, no such studies have been carried out in thalamus. Some recent studies using genetically manipulated mice suggest particular subtypes that are involved in sedation, and these are detailed in Section 6.5. The anatomical distribution of GABA_A receptor subtypes is quite specific and is likely to be related to their individual roles within the CNS. Within the hypothalamic pathway no specific studies have been carried out to investigate which subtypes are involved; however, based on in situ hybridization and autoradiographical studies, it is likely that α_1 and α_2 subtypes play a predominant role, together with β_2 and γ_2 [68]. The thalamus is highly discrete in terms of which GABA receptor subtypes are expressed. In the major part of the thalamus, the ventrobasal VB nucleus, there are two major subtypes: $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_{2/3}\delta$ [68, 69]. These subtypes are also expressed differently at the cellular level, since $\alpha_1\beta_2\gamma_2$ is predominantly located at inhibitory synapses, whereas $\alpha_4\beta_{2/3}\delta$ is located extrasynaptically on neuronal cell soma [70]. The reticular nucleus expresses a completely different type of receptor, being predominantly $\alpha_3\beta_3\gamma_2$ [68]. All these subtypes possess different pharmacological and biophysical properties indicating that they subserve different physiological roles and making them amenable to the development of selective drugs [71].

6.4 THE DEVELOPMENT OF SAFER AND MORE SELECTIVE HYPNOTICS

Over the last century the development of sedative drugs has evolved through several phases but has produced some of the most highly prescribed medicines in history. Some of the major drugs and their targets are summarized in Table 6.1. The first synthetic depressant to be used for treating insomnia was chloral hydrate [72]. It was first synthesized in 1832 and is amazingly still in limited use today. It was originally used to treat alcoholics with disturbed sleep and patients with chronic pain who could not sleep. Administering the drug to alcoholics was quite dangerous since chloral hydrate interacts strongly with alcohol: a combination of these two is commonly known as a “Mickey Finn” and produces a powerful “knockout” effect [73]. Like alcohol, chloral hydrate is also addictive and tolerance to the drugs effects occur on extended use. In addition, the drug is carcinogenic [74]. Chloral hydrate is metabolized rapidly to the active component trichloroethanol (TCE), which is known to interact with several ligand-gated channels, including GABA_A, glycine, and NMDA receptors [75, 76].

6.4.1 Barbiturates

The group of drugs that really first popularized the market for treatment of insomnia comprised the barbiturates. Synthesized in 1903 diethyl barbituric acid was the first orally available barbiturate, known as Veronal. This drug was a highly effective sedative, but the effects were very long-lasting and next-day drowsiness was a major

TABLE 6.1 Sedative/Hypnotic Drugs

Class	Trade Name	Dose (mg)	Receptor Target	Status	Issues
Chloral hydrate	Noctec	500–2000 mg	GABA _A / <i>N</i> -methyl-D-aspartate (NMDA) receptors	Low usage	Gastrointestinal upset and diarrhea, tolerance
Barbiturates					
Amobarbital	Amytal	65–200	GABA _A receptors	Relatively low usage for insomnia, some use prior to surgery	Low therapeutic index, tolerance abuse liability
Phenobarbital	Luminal	30–300			
Secobarbital	Seconal	100			
Pentobarbital	Nembutal	100			
Barbiturate-like					
Ethylchlorynol	Placidyl	500–1000	Unknown	Very little use due to low therapeutic index and abuse	Toxicity problems, tolerance and abuse potential, now considered obsolete
Glutethimide	Doriden	250–500			
Methylprylon	Noludar	200–400			
Methaqualone	Quaalude	150–300			
Meprobamate	Equanil, Miltown	1200–1600			
Benzodiazepines					
Diazepam	Valium	5–20	GABA _A receptor potentiation; show some selectivity for the α ₁ -containing subtype of GABA _A receptors	Prescription drug of choice for short-term insomnia, very safe and has high usage but limited for long-term treatment	Abuse potential, tolerance
Alprazolam	Xanax	0.25–0.5			
Clonazepam	Rivotril	0.25–2			
Flunitrazepam	Rohypnol	1–2			
Lorazepam	Ativan	2–4			
Triazolam	Halcion	0.1–0.25			
Other BZ site drugs					
Zolpidem	Ambien	5–10			
Zaleplon	Sonata	5–10			
Zopiclone	Imovane	7.5			

(Continued)

TABLE 6.1 (*Continued*)

Class	Trade Name	Dose (mg)	Receptor Target	Status	Issues
Antihistamines					
Diphenhydramine	Benadryl	25–70	Histamine receptors	Commonly used over-the-counter medicine for mild insomnia	Mild effects, long-lasting drowsiness, dry mouth
Hydroxyzine	Atarax, Vistaril	50			
Triazolopyridines					
Trazodone	Trazorel/Desyrel	50–100	Serotonin 2A and 2C receptor antagonist	Used for treating depression induced insomnia	Hypotension, stomach upset, dry mouth
Nefazodone	Serzone	200–600			
Melatonin	Melatonin	2–10	Melatonin receptor agonist	Sold as a “dietary supplement;” thought to affect circadian clock	Poor clinical evidence of effectiveness

problem. In 1912, phenobarbital was introduced to treat anxiety and insomnia. In addition to its sedative and hypnotic properties, phenobarbital was an effective anticonvulsant and has become one of the most important pharmacological treatments for epilepsy. The highly efficacious nature of barbitals and phenobarbital made these drugs very successful, spawning the synthesis of over 2500 different barbiturates [77]. The effects of these various barbiturates are generally similar, differing primarily in potency, duration of action, and route of administration, some being developed as anesthesia induction agents. The use of barbiturates to treat anxiety and insomnia boomed in the 1950s and grew to a peak in the 1960s despite growing fears regarding addiction liability and a very narrow margin between effective doses and those that produced serious side effects and death [78]. In the United Kingdom alone in 1966 there were over 16 million prescriptions for barbiturates. Growing concern among doctors regarding the abuse and safety of barbiturates together with the availability of the benzodiazepines eventually resulted in reduced use of these drugs [79], and orally administered barbiturates are not currently routinely prescribed for insomnia, although rapid-acting intravenous forms are still utilized to a high degree in anesthesia [80]. Barbiturates act by binding to the GABA_A receptor and enhancing inhibitory neurotransmission in the CNS [81]. As mentioned above, they appear to have a nonselective mechanism with activity at the majority of GABA_A receptor subtypes [82].

6.4.2 Barbiturate-Like Drugs

With the popularity in the use of barbiturates a number of companies developed novel “tranquilizers” which behaved in a similar fashion but were not barbiturate-like molecules. These included ethchlorvynol (Placidyl), glutethimide (Doriden), methylprylon (Noludar), meprobamate (Miltown), and the quinazolones, such as methaqualone (Quaalude) [83]. Unfortunately, despite initial promise, these drugs did not differ from barbiturates in their safety profile, tolerance effects, or propensity to be abused and were not really considered to be an advance in any way. Quaaludes in particular became highly abused in the late 1970s and were one of the most popular recreational drugs of the time [84]. As a consequence they were prescribed with decreasing frequency when safer sedatives became available and are now essentially obsolete.

6.4.3 Benzodiazepines

The popularity of the barbiturates indicated a huge demand for novel anxiolytic and sedative drugs. The highlighted risks with barbiturates and related compounds, especially the low therapeutic window, meant that doctors were becoming more reluctant to prescribe these particular drugs. In 1954 a scientist at drug company Hoffman La Roche synthesized a compound which seemed to be pharmacologically inert. After spending three years on a shelf in the laboratory, the compound was submitted for screening and found to have sedative/hypnotic effects in animals but was much more potent than the barbiturates and safe to administer at high doses [85]. This compound (chlordiazepoxide) was developed and was launched on the market in 1960 as Librium, the first benzodiazepine sedative. The more potent diazepam Valium followed in 1963, and many other related benzodiazepines

followed these. This class of compound was much more potent than previous sedatives and did not have the associated toxicity problems or liabilities on overdose. The benzodiazepines became some of the most successful drugs in history with record prescriptions in the 1970s and 1980s [86]. While the toxicity problems were not an issue, it became apparent that the tolerance and abuse potential seen with barbiturates were still present in the benzodiazepines [87]. Also, while effective treatments for insomnia, benzodiazepines often produced next-day sedation, ataxia, and memory and cognitive impairment [88, 89]. As a result the usage of benzodiazepines for longer term treatment has declined in recent years; however, they are still very effective and fast-acting drugs for short-term use.

As detailed above, in the late 1980s, GABA_A receptors were found to be made up of a variety of subtypes that differed in pharmacology and biophysical properties. It was found that one of the more recent benzodiazepine receptor insomnia agents, zolpidem, demonstrated some selectivity for the α_1 subtype and was relatively more effective as a hypnotic agent than an anxiolytic [90, 91]. Zolpidem has become one of the leading current therapies for insomnia, and similar “nonbenzodiazepine” benzodiazepine ligands such as zaleplon and the soon to be launched indiplon behave in a similar fashion with a small degree of α_1 selectivity [92, 93]. As described below, genetic mouse models have recently identified the α_1 -subtype as having a major role in the sedative properties of benzodiazepines.

6.4.4 Non-GABA_A Receptor Agents

In the last few years, several of the serotonin (5-HT)–modulating antidepressants such as trazodone and nefazodone, which produce sedation as a side effect, have been prescribed for insomnia, especially in depression-related insomnia [94, 95]. These drugs are relatively safe, with no issues regarding tolerance or abuse potential, and probably act via their effects as antagonists at 5-HT receptors.

The hormone melatonin is intrinsic to the control of rhythmic circadian activity in the brain and is released from the pineal gland during the nighttime. Low levels of melatonin have been linked to insomnia and administration of melatonin is believed to be sleep promoting [96]. The mild hypnotic properties of melatonin and its availability as a “natural supplement” have led to widespread use of the agent for insomnia [97]. However, evidence of the efficacy of melatonin is based on limited clinical trial data. Furthermore, melatonin has some properties that would be expected to limit its usefulness as an oral agent, such as short half-life and high first-pass metabolism.

Mild insomnia can also be treated using antihistamines, which produce some degree of sedation. There are many over-the-counter treatments such as Benadryl, Sleep-Eze, and Nytol that contain diphenhydramine or hydroxyzine and will give temporary relief for sleep problems [98]. These agents do tend to cause daytime drowsiness and are not recommended for continuous use since they also exhibit tolerance [99].

6.4.5 Potential New Therapies

There are a number of new therapeutic agents in development for treatment of insomnia and sleep-related disorders. Table 6.2 lists these candidates and gives some

TABLE 6.2 Novel Therapeutics Currently in Development

Class	Trade Name	Receptor Target	Company	Status
Benzodiazepine site				
Eszopiclone	Lunesta	GABA _A receptor potentiation; eszopiclone is a nonselective BZ site agonist similar to diazepam; indiplon, like zolpidem, has some selectivity for the α_1 subtype	Aventis	Launched April 2005
Indiplon	Indiplon		Dov/Neurocrine/Pfizer	Phase III
NGD-2-73			Neurogen	Phase II
GABA agonist: gaboxadol (THIP)		GABA _A receptor agonist, selective for δ -containing receptors	Lundbeck/Merck	Phase III; expected launch in 2007
Melatonin				
Ramelteon (TAK-375)	Rozerem	Melatonin receptor agonists	Takeda	Launched September 2005
LY-156735			Lilly	Phase II
Serotonin receptor				
APD-125		5-HT _{2A} receptor antagonists	Arena	Phase I
EMD-281014			Merck KGaA/Lilly	Phase I
eplivanserin			Sanofi-Aventis	Phase II
Mixed: doxepin		Histamine antagonist; muscarinic ACh antagonist	Somaxon	Phase III

indication of their mechanism and anticipated launch on to the market. The most imminent compounds are Lunesta (eszopiclone, an isomer of zopiclone; launched April 2005 [100] and indiplon, both benzodiazepine modulators. Similar to zolpidem, indiplon, shows some α_1 -subtype selectivity [101]. Lundbeck and Merck is developing gaboxadol (also known as THIP) [102], a GABA receptor agonist which demonstrates selectivity for the $\alpha_4\beta_\delta$ subtype of the GABA receptor [103]. The presence of this subtype, which is insensitive to benzodiazepines in thalamus, targets a different mechanism to that of the available benzodiazepine site drugs such as zolpidem, and evidence suggests that this drug increases slow-wave sleep with no effect on REM, having the potential for fewer side effects [104]. Another target which seems to increase slow-wave sleep is the 5-HT_{2A} receptor [105], and several compounds are in early phases of development (see Table 6.2). The role of 5-HT_{2A} receptors in sleep pathways is not yet clearly understood, and further work is necessary to understand the mechanism of action of these compounds. The effects of melatonin have prompted development of more potent and brain-penetrant melatonin agonists, and several compounds are being developed. The closest of these to launch is Ramelteon, which is currently in phase III trials [106].

6.5 GENETICALLY MODIFIED MICE AS TOOLS TO UNDERSTAND FUNCTION OF HYPNOTICS/SEDATIVES

The use of so-called knockout mice, where a gene is specifically functionally deleted from the animal's genome, has proven a valuable approach to defining the functional and physiological role of that gene product. However, such an approach has two well-known caveats that can compromise interpretation of the phenotype: developmental effects (i.e., the effect of deleting the gene from conception affects the phenotype of the adult due to effects on the development of the animal) and compensation (the alteration in the expression of other genes to compensate for the loss of expression of the targeted gene). Indeed, in the case of the GABA_A receptor family, examples of compensation have been shown to occur [107]. However, for the GABA_A receptor, it has been possible to bypass these two concerns through the elegant use of a combination of genetically engineered mice and pharmacology.

Site-directed mutagenesis studies discussed above revealed the key amino acids that determined benzodiazepine binding to the GABA_A receptor. A key residue is histidine 101 of the α_1 subunit. The presence of a histidine residue at position 101 of the primary amino acid sequence of the α_1 subunit (numbering for the α_1 subunit, but the equivalent amino acid exists for α_2 , α_3 , and α_5 subunits) confers high-affinity binding of diazepam, the presence of an arginine (in α_4 and α_6 subunits) conferred low affinity [108]. The amino acids are interchangeable between the subunits, conferring the appropriate affinity for diazepam without affecting any other property of the receptor. Recapitulating this observation in an animal would open up the possibility of making a mouse with a particular GABA_A receptor subtype that was diazepam insensitive but otherwise wild type (WT) in its functionality, thereby avoiding the issues of developmental effects/compensation. Using the transgenic technology of homologous recombination in mouse embryonic stem cells, it is indeed possible to generate such a "knockin" mouse, where a single amino acid change is been introduced into the genome. Two groups have now taken this approach, in the

first instance generating a genetically modified mouse where amino acid residue 101 of the GABA_A receptor α_1 subunit has been changed from a histidine to an arginine, and subsequently generating the cognate mice where the equivalent histidines in the α_2 , α_3 , and α_5 subunits are mutated to arginine [109–111]. When used in combination with benzodiazepine site ligands affected by the mutation, these mice are powerful tools to investigate the physiological role of individual receptor subtypes.

Initial studies with $\alpha 1\text{His}101\text{Arg}$ mice demonstrated that they were much less sensitive to the sedative/hypnotic effects of diazepam than WT animals, indicating that $\alpha_1\beta\gamma_2$ -containing GABA_A receptors mediated the sedative effects of benzodiazepines [109, 110]. This was not unexpected, given that the α_1 -subtype selective drug zolpidem is a potent hypnotic. Indeed, subsequent studies with zolpidem in the $\alpha 1\text{His}101\text{Arg}$ mice confirmed that its sedative effects are mediated through α_1 -containing GABA_A receptors [112]. More detailed studies employing EEG analysis have explored the effects of diazepam on sleep architecture. It was noted that activity of diazepam at α_1 -containing receptors was not necessary for the effects of diazepam on latency to sleep, the amount of sleep, or the effects on REM sleep. Interestingly, other parameters, such as the suppression in slow-wave sleep by diazepam, were actually enhanced in the $\alpha 1\text{His}101\text{Arg}$ mice [113]. These data suggested to the authors that the effects of diazepam on sleep are mediated by receptor subtypes other than α_1 (i.e., α_2 -, α_3 -, or α_5 -containing receptors) and that the hypnotic effect (and the effect on sleep architecture) can be separated from the sedative activity. The role of α_3 -containing receptors in mediating the effects of diazepam on sleep architecture was similarly explored using $\alpha 3\text{His}105\text{Arg}$ mice. The effects of diazepam on EEG were the same in both the $\alpha 3\text{His}105\text{Arg}$ and WT mice [114]. Thus α_3 -containing GABA_A receptors do not appear to mediate the effects of diazepam on sleep architecture. Most recently this same group have used similar techniques in $\alpha 2\text{His}100\text{Arg}$ mice and found that the suppression of delta power by diazepam was reduced in these mice [115]. In addition diazepam enhancement of theta activity was also absent in the mutant mice. These findings suggest that the α_2 -containing receptors, possibly those in the hypothalamic sleep pathway, mediate some of the effects of benzodiazepines on sleep EEG patterns.

The contribution of different β subunits has also been investigated recently using mutant mice based on the sedative and anesthetic properties of etomidate. This compound is selective for β_2 - and β_3 -containing receptors and dependent on an asparagine residue in the transmembrane domain [116]. Mutation of this residue to Ser in β_2 subunits rendered these mice etomidate sensitive at only β_3 . Etomidate was still anesthetic in these mice, but they were not sensitive to sedative doses, suggesting that β_2 -containing receptors produced the sedating effects but not the anesthetic properties of etomidate [117]. A complimentary study using β_3 -mutated mice demonstrated that the anesthetic properties of etomidate such as loss of righting and withdrawal reflex are mediated via β_3 -containing receptors [118]. These data show that sedative and anesthetic properties can be separated and are controlled through different receptor subtypes. Sleep states were also investigated in the β_3 knockout mice which show a major disruption in thalamic circuitry [119]. In these mice the timing of sleep states was similar to WT but EEG delta power was dramatically increased in non-REM sleep. Reduction of delta power by midazolam was present in both WT and knockout mice. From this study it is clear that some aspects of sleep are affected by loss of β_3 but others, including the reduction of delta

by benzodiazepines, are not. With the caveat mentioned above regarding possible compensatory changes, hypnotics have also been investigated in α_1 -, β_2 -, and δ -subunit knockout mice. Deletion of the α_1 or β_2 subunit resulted in reduced sensitivity to the hypnotic effects of benzodiazepines and the GABA agonist gaboxadol. The β_2 knockout mice were also less sensitive to etomidate [120]. In the δ -subunit knockout mouse sleep time induced by benzodiazepines and some anaesthetics was unaffected, but neurosteroid-induced sleep time was significantly reduced, suggesting the sedating properties of neurosteroids may be mediated via this subtype [121]. One question that had not been addressed in any of the above studies was the induction of tolerance to the sedative effects of diazepam. The knockin mice represent an ideal tool to explore the molecular mechanism of tolerance, and indeed van Rijnsoever et al. have performed such a study. They found that, while WT, α_2 and α_3 knockin mice developed tolerance to the sedative effects of diazepam (the α_1 knockin mice were unaffected by diazepam), the α_5 knockin mice did not [122]. These data suggest that chronic activation of α_5 -containing GABA_A receptors is necessary for the development of tolerance to the sedative effects of diazepam.

6.6 FUTURE DIRECTIONS

Despite the limited use of some of the older sedatives the progression has been to utilizing new, lower liability compounds as hypnotics. However, there are still clearly perceived risks of tolerance and dependence to these compounds if they are used for extended periods of time. The clinical need is for highly effective agents with a low potential for addiction and tolerance, with no cognitive or sedative “hangover” side effects. Improved pharmacological understanding of GABA subtypes and the utility of transgenic mice have enabled much progress in understanding the mechanisms of sleep and sedation, but we now need to understand better the processes that lead to insomnia to develop improved drugs. There are still many questions to be answered. What links insomnia and poor daytime performance/memory? What is the link with many of the GABA hypnotics and dependence? Do the current therapeutics really address an underlying mechanism? How do we impact upon “quality of sleep”? Hopefully these are now really addressable questions and will lead to a new generation of sleep medicine.

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REGULATION OF ADULT NEUROGENESIS

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7.1	Multiple Regulation Points	204
7.2	Regulation of Survival	204
7.3	Proliferation	206
7.3.1	Running	206
7.3.2	Stress/Corticosteroids	206
7.3.3	Sex Steroids	207
7.3.4	Glutamate	207
7.3.5	Cholinergic System	208
7.3.6	Serotonin and Antidepressants	209
7.3.7	Psychotropic Drugs	210
7.3.8	Neuropeptides and Growth Factors	210
7.4	Conclusions	211
	Acknowledgment	212
	References	212

The vast majority of neuronal precursors in the mammalian brain undergo terminal differentiation and become unable to divide once development is complete. However, it has been shown that several neuronal populations continue to be generated from dividing precursor cells well into adulthood. Dentate gyrus granule cells, the primary neurons of this portion of the hippocampus, are born from precursors residing within the dentate gyrus itself [1–3]. In contrast, olfactory bulb granule cells are generated by precursor cells in the subventricular zone (SVZ) and then migrate along the rostral migratory stream into the olfactory bulb [4, 5]. Calretinin-expressing striatal interneurons are also born in adulthood, most likely from precursor cells within the SVZ [6]. Finally, new cortical neurons appear to be generated from precursor cells that are located within the adult cortex [6–8]. This review will discuss factors that regulate the generation of new neurons in the adult nervous system, focusing primarily on the dentate gyrus and olfactory bulb granule cells since little is known

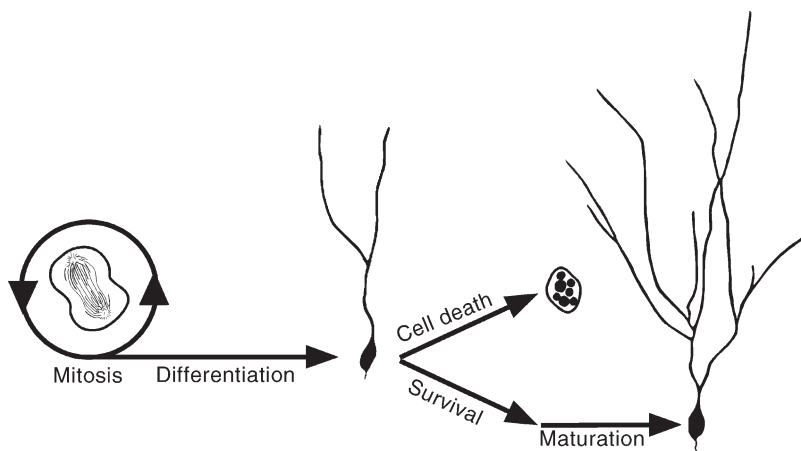


Figure 7.1 Generation of new neurons in adulthood can be divided into separate processes that are independently regulated. Differentiation and maturation appear to occur constitutively; that is, no factors have been shown to enhance or disrupt either the generation of neurons from new daughter cells or the maturation of neurons that do not die. Production of new cells through proliferation of precursor cells is highly regulated in the dentate gyrus but appears to be regulated by fewer factors in the SVZ. Cell death, or survival, is closely regulated in both regions.

about the regulation of adult neurogenesis in other regions. Understanding the roles of extrinsic factors in adult neurogenesis may suggest links between dysregulation of neurogenesis and disease states, such as depressive and seizure disorders, and may provide clues for treating pathological conditions involving loss of neurons.

7.1 MULTIPLE REGULATION POINTS

There are several processes involved in neurogenesis that appear to be regulated independently. These include proliferation of precursor cells, differentiation and maturation of new daughter cells into neurons, and survival of young neurons (Fig. 7.1). There are a few reports of altered proportions of new neurons and glial cells being generated due to different genotypes, treatment with growth factors, blockade of calcium channels, or enrichment of housing conditions [9–12]. These alterations in phenotype could reflect regulation of differentiation by these factors but could also result from selective proliferation of cell-type-specific progenitor cells or selective death of cells belonging to one phenotype.

7.2 REGULATION OF SURVIVAL

Under normal laboratory conditions, approximately half of the new granule neurons in the rat hippocampus and olfactory bulb die within one and three months, respectively [13–15]. New neurons that survive this one- or three-month period are rarely lost over the next year [13–15]. Survival of young granule cells is highly dependent on environmental variables. Treatment differences seen at early survival time points can be lost after longer survival, indicating that additional granule cells

generated by higher rates of proliferation have all died [16, 17]. This loss of extra cells suggests that the number of granule cells that survives reflects the number that is used or needed, rather than a fixed proportion of the number generated.

Use-dependent survival of young granule cells is also suggested by several of the factors that regulate survival. Multiple studies have also shown a positive effect of enriched housing, when compared with standard laboratory housing, on hippocampal granule cell survival [11, 18–23]. The enrichment in these studies included larger cages, more cagemates, and novel objects; it is not clear which of these features are key to the changes in granule cell survival [19], but all have the potential to provide increased opportunity for learning experiences for the animals. Interestingly, the enrichment features that increase dentate gyrus granule cell survival have no effect on young granule cells in the olfactory bulb [18]; however, odor enrichment, that is, increased exposure to novel odors, does increase survival of olfactory bulb granule cells [24–28]. In birds, housing environments with more complex social groups increase survival of new neurons in three regions involved in vocal communication [29], further strengthening the idea that system-specific stimuli increase new neuron survival.

Hippocampus-dependent learning alters survival of new hippocampal neurons. Training in hippocampus-dependent versions of the Morris water maze or eyeblink classical conditioning has been shown to increase survival of one- to two-week-old granule cells [30, 31]. Other studies have found decreases or no change in young neurons with water maze training [32–34], suggesting that subtle differences between the experimental paradigms, such as the timing and number of training trials or the age of the young cells examined, may determine the specific effect of learning on granule cell survival. A recent study using a hippocampus-dependent olfactory association task, social transmission of food preference, found increased granule neuron survival after one day of training and decreased survival with additional training, suggesting that new granule cells may be initially saved but then actually killed by learning [35].

The increase in dentate gyrus granule neuron survival with learning is not due to learning-related stress [30, 35]. In fact, stress would be expected to have the opposite effect, since injection of corticosterone decreases granule neuron survival and removal of corticosteroids by adrenalectomy increases survival [36]. Corticosteroids may act directly on new granule cells to alter their survival, as most young granule neurons express glucocorticoid receptors and/or mineralocorticoid receptors within a few days of their birth [37, 38]. Another steroid hormone, estrogen, increases survival of new dentate gyrus granule neurons in male meadow voles when given 6–10 days after injection of the cell division marker bromodeoxyuridine (BrdU) but not 5 days earlier or 5 days later [39]. Testosterone has been shown to increase survival of new neurons in the adult female canary brain [40]; however, its role in adult neurogenesis in the mammalian brain is unclear [41, 42].

Learning-related increases in survival of young dentate gyrus granule cells could be mediated by the cholinergic system. Loss of cholinergic input from the basal forebrain to the hippocampus impairs hippocampus-dependent learning [43, 44] and decreases neurogenesis in the dentate gyrus [45]. Increased numbers of dying cells found in the dentate gyrus subgranular zone after basal forebrain lesion [45] suggest that new neuron survival is decreased by loss of cholinergic input. It is difficult to determine whether proliferation is also changed, due to the long survival time after BrdU injection in this study. While this finding shows a correlation between decreased

survival of new neurons and impairment of hippocampal function, loss of cholinergic input to the dentate gyrus could impair learning by inhibiting granule cell survival, or the loss of input could inhibit granule cell survival by impairing learning (see above).

One paracrine factor that appears likely to increase survival of new neurons, based on its functions *in vitro*, is brain-derived neurotrophic factor (BDNF) [46]. In the canary high vocal center (HVC), BDNF infusion has clearly been shown to increase survival of new neurons during a discrete time period between 14 and 20 days after the birth of the new cells. BDNF infusion beginning 10 days earlier or 10 days later has no effect [47–49]. Infusion of anti-BDNF antibodies decreases neuronal survival, demonstrating that endogenous BDNF functions as a survival factor in this system [49]. In the mammalian brain, the specific action of BDNF is less clear. Although infusion of BDNF increases the number of BrdU-labeled neurons in the dentate gyrus, olfactory bulb, and striatum [50–53], the timing and number of BrdU injections make it difficult to determine whether these changes reflect increased cell proliferation or increased survival of new neurons. Two antidepressants that have been shown to increase BDNF levels in the hippocampus, fluoxetine and rolipram [54–56], also increase survival of new neurons in the dentate gyrus [57]. Interestingly, 13-*cis*-retinoic acid (Accutane), which has been reported to produce symptoms of depression in some people, decreases survival of new granule cells in the adult dentate gyrus [58, 59].

7.3 PROLIFERATION

7.3.1 Running

Several studies have found that running increases neurogenesis in the dentate gyrus, but not in the olfactory bulb, of adult rodents [18, 60]. Increased numbers of BrdU-labeled cells have been seen at short survival times after BrdU [61, 62], with staining for the endogenous marker of mitosis Ki67 [61], and with increased numbers of cells expressing a marker of immature neurons [63], all indicating that the effect of running is on proliferation. Although survival effects have not been directly examined, the proportional increase in newborn cells after long survival periods does not increase, suggesting that running does not affect survival of new neurons [18, 19, 60]. Forced running (on a treadmill) and voluntary running, that is, access to a running wheel in the cage, both increase proliferation [61, 62]. The mechanism underlying this running-induced increase in granule cell precursor proliferation is unclear; several factors have been proposed, including insulin-like growth factor (IGF) [64], vascular endothelial growth factor (VEGF) [65], and *N*-methyl-D-aspartate (NMDA) receptor subunits containing the NR2A subunit [66].

7.3.2 Stress/Corticosteroids

Proliferation of granule neuron precursors in the adult brain is strongly regulated by glucocorticoid hormones [67–69]. Removal of endogenous glucocorticoids by adrenalectomy increases cell proliferation, indicating that endogenous corticosterone normally slows proliferation [67, 69]. High glucocorticoid levels, produced either by direct injection of corticosterone or by the adrenal glands in response to a stressful experience, strongly inhibit granule cell precursor proliferation [17, 67, 70]. Acute and chronic stress from several different types of stressors, including physical

restraint, predator odor, and psychosocial stress, all have been shown to inhibit proliferation [17, 70–74]. Stress-induced inhibition of proliferation has been observed in rats, tree shrews, and primates. Interestingly, acute restraint stress in mice appears to increase cell proliferation [75]. Glucocorticoids do not alter cell proliferation in the SVZ [76]. However, a two-week treatment with corticosterone strongly inhibits proliferation of neocortical cells expressing the chondroitin sulfate proteoglycan NG2 [76], the cells that are thought to be the precursors for adult-generated neocortical interneurons [6]. However, since only a small percentage of newborn cells in the adult cortex appear to generate neurons [6], changes in proliferation of NG2-expressing cells do not necessarily indicate changes in cortical neurogenesis. Both mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) appear to be involved in the regulation of proliferation in the dentate gyrus [77]. But no dividing cells express detectable MR and only 13% express GR [37, 38], indicating that corticosteroid regulation of proliferation is likely to be indirect. Aging is associated with increased glucocorticoid levels [78, 79] and decreased proliferation of dentate gyrus granule cell precursors [11, 80–82]. This decrease can be reversed by adrenalectomy in old age [83]. In the SVZ, a recent study found a dramatic decrease in cell proliferation with age [84], while an older study found no difference [80]. Species and/or sex differences may explain these contradictory results, since the former study used male C57BL/6 mice, while the latter used female Fisher 344 rats.

7.3.3 Sex Steroids

Cell proliferation in the female rat dentate gyrus is increased by acute systemic injection of estrogen [16, 39, 85]. Interestingly, chronic treatment with estrogens results in no change, or even a decrease, in granule cell precursor proliferation [39, 85], while chronically low levels of estrogens decrease the responsiveness of the precursors to estrogen replacement, resulting in a permanent inhibition of neurogenesis [85]. Endogenous estrogen also increases proliferation, as shown by the peak in granule cell precursor proliferation during the 12-hour-long proestrous phase of the estrous cycle, when estrogen levels are highest [16]. Reproductively active female voles have decreased levels of cell proliferation in the dentate gyrus relative to reproductively inactive females, which may be related to estrogen levels [39]. Somewhat paradoxically, pregnant mice do not appear to have altered levels of cell proliferation in the dentate gyrus but show increased proliferation in the SVZ. However, this pregnancy-induced increase in SVZ cell proliferation can be mimicked by prolactin, not estrogen [86]. Estrogen receptor α has been observed within the dentate gyrus but does not appear to be present in dividing precursors, while estrogen receptor β is not present in the dentate gyrus at all, suggesting that the effects of estrogen on dentate gyrus granule cell precursors are not direct [85].

The testosterone analog nandrolone (19-nortestosterone) decreases granule cell precursor proliferation in the adult dentate gyrus [41]. Androgen receptors are plentiful in the dentate gyrus granule cell layer [41], but it is not known whether they are present in dividing cells.

7.3.4 Glutamate

Granule cells in the adult dentate gyrus receive their major input through the perforant path from glutamatergic neurons in the entorhinal cortex [87]. Disruption

of this input in adulthood by a lesion of the entorhinal cortex increases cell proliferation in the dentate gyrus [88]. Interestingly, stimulation of the glutamatergic mossy fibers, the axons of the granule cells, also increases neurogenesis in this region, although it is unclear whether proliferation or survival of the new granule cells is affected [89].

Glutamate acts through four major types of receptors: NMDA receptors, α -amino-3-hydroxy-methylisoxazole-4-propionic acid (AMPA) receptors, kainate receptors, and metabotropic receptors (reviewed in [90]). Competitive and noncompetitive NMDA receptor antagonists, including CGP 37849, MK-801, and ketamine, significantly increase the number of S-phase cells in the dentate gyrus within 3 h of systemic injection [68, 88, 91, 92]. Many of the extra cells generated after a single injection of an NMDA receptor antagonist survive for four weeks and mature into granule neurons, resulting in a significant increase in the total number of granule neurons in the granule cell layer [88]. Activation of NMDA receptors, by systemic injection of NMDA, significantly inhibits dentate gyrus granule cell proliferation [88].

Somewhat paradoxically, AMPA receptor activation has the opposite effect of NMDA receptor activation; potentiation by injection of LY451646, an allosteric positive modulator of AMPA receptors, increases cell proliferation in the dentate gyrus [93]. Increased proliferation is seen after acute (single-injection) and chronic (21-day-injection) treatment with LY451646 [93]. Interestingly, these opposite effects of NMDA and AMPA receptor activation on proliferation parallel antidepressant actions of drugs acting on these receptors. NMDA receptor antagonists and AMPA receptor potentiators show antidepressant activity in humans and/or animal models [94, 95]. Interestingly, acute doses of both NMDA receptor antagonists and AMPA receptor potentiators rapidly change cell proliferation as well as mood in humans or behavior in animal models of depression [88, 93, 96, 97].

Chronic inhibition of group II metabotropic receptors, with the antagonist MGS0039, increases granule cell precursor proliferation [98] and shows antidepressant-like effects in two animal models of depression, the tail suspension test and the forced-swim test [99]. These antidepressant-like effects of the metabotropic glutamate receptor antagonist appear to be mediated through the AMPA receptor, as they can be blocked by NBQX, an AMPA receptor antagonist [100].

Systemic injection of kainate causes a large increase in cell proliferation in the dentate gyrus [101–103]. However, it is unclear whether this effect is due to activation of the kainate receptor itself or to the resulting cell death or other damage caused by the seizures kainate produces, since discrete lesions of the granule cell layer are known to stimulate proliferation of nearby granule cell precursors [104]. Interestingly, kainate-induced seizures decrease rather than increase granule cell precursor proliferation in rodent pups, apparently by increasing glucocorticoid levels [105].

Glutamate does not appear to act directly on NMDA receptors on precursor cells, since dividing cells in the adult dentate gyrus show no detectable immunoreactivity for the critical NMDA receptor subunit NR1 [106, 107]. The possibility that other types of glutamate receptors are expressed by dividing cells has not been examined.

7.3.5 Cholinergic System

Two studies using transgenic mice have shown that partial or complete reduction of α_7 and β_2 nicotinic cholinergic receptors decreases proliferation of granule cell

precursors in the adult dentate gyrus [108, 109]. However, injection of nicotine itself inhibits granule cell precursor proliferation in the dentate gyrus of juvenile, one-month-old, rats [110]. These apparently contradictory results could result from developmental effects of nicotinic receptor knockout or from nonspecific effects of nicotine.

7.3.6 Serotonin and Antidepressants

Acute treatment with serotonin (5-HT) type-1A receptor antagonists NAN-190, *p*-MPPI, and WAY-100635 decreases proliferation in the adult dentate gyrus by 30% within 2.5 h of systemic injection [111]. Acute (4-h) and chronic (28-day) treatment with the 5-HT_{1A} agonist 8-OH-DPAT increase granule cell precursor proliferation in the adult dentate gyrus [112, 113]. 5-HT_{1B} receptor activation and inhibition by sumatriptan and GR 127935, respectively, have no effect on normal rats [112]. However, sumatriptan can reverse the decrease in proliferation seen with 5-HT depletion by the synthesis blocker parachlorophenylalanine [112]. Blockade of 5-HT_{2A} receptors inhibits proliferation in the adult dentate gyrus, although receptor activation has no effect. Neither activation nor inhibition of 5-HT_{2C} receptors alters cell proliferation in the dentate gyrus. The antidepressant medication fluoxetine, which acts as a specific serotonin reuptake inhibitor (SSRI), also increases granule cell precursor proliferation [113, 114]. Interestingly, although 5-HT receptor agonists/antagonists rapidly alter granule cell precursor proliferation, fluoxetine is only effective after chronic treatment. Treatment with fluoxetine for 7 days or less has no discernable effects on cell proliferation, while treatment for 11 days or longer results in significant increases in division of granule cell precursors [113–115]. The reason for this difference in the time course of treatment necessary to increase cell proliferation is not clear. The majority of the studies of antidepressant effects on adult neurogenesis have examined cell proliferation several days after antidepressant treatment was stopped, suggesting that chronic treatment may be necessary in order to see a long-lasting effect of treatment. However, one study in which BrdU was given only 3 h after the final antidepressant dose also failed to find any effect of subchronic (7-day) treatment [115].

In the SVZ, 5-HT receptor agonists and antagonists alter neuronal precursor proliferation in ways that are similar, though not identical, to the changes observed in the dentate gyrus. 5-HT_{1A} and 5-HT_{2C} receptor agonists rapidly increase SVZ cell proliferation, while 5-HT_{1B} receptor activation inhibits cell proliferation [112]. Interestingly, even chronic treatment with the SSRI fluoxetine has no effect on cell proliferation in the SVZ [115].

In addition to fluoxetine, a large number of other antidepressants, with widely varying mechanisms of action, have been shown to increase granule cell precursor proliferation in the adult dentate gyrus. These include the tricyclic antidepressant imipramine [113], the monoamine oxidase inhibitor tranylcypromine [114], the specific norepinephrine reuptake inhibitor reboxetine [114], the phosphodiesterase-IV inhibitor rolipram [116], and electroconvulsive seizure therapy [114, 117]. Like fluoxetine, rolipram required chronic treatment in order to increase precursor proliferation [116]; however, increased cell proliferation was seen following a single electroconvulsive seizure [117]. Conversely, 13-*cis*-retinoic acid, which can cause symptoms of depression, decreases cell proliferation in the dentate gyrus [58].

Clozapine and haloperidol, which are antipsychotic rather than antidepressant drugs, have no effect on cell proliferation in the dentate gyrus or SVZ [114, 118]. A comprehensive review of the effects of antidepressants on adult neurogenesis has recently been published by Malberg and Schechter [119].

7.3.7 Psychotropic Drugs

Chronic opiate treatment inhibits granule cell precursor proliferation in the dentate gyrus, whether it is self-administered (heroin, 26 days) or injected/implanted (morphine, 5–7 days) [120–122]. Interestingly, withdrawal from morphine for one week, after one-week treatment, results in a rebound twofold increase in cell proliferation [121]. Acute treatment (morphine, a single intraperitoneal injection) has no effect on dentate gyrus cell proliferation [120]. However, a single injection of the μ -opioid receptor antagonist naltrexone inhibits granule cell precursor proliferation [123]. In the adult SVZ, neither acute nor chronic morphine affects cell proliferation [120]. In rat pups, acute naltrexone increases cell proliferation in the SVZ [124], the opposite of what has been observed in the adult dentate gyrus. Like opiates, high-dose ethanol decreases dentate gyrus granule cell precursor proliferation, while subsequent withdrawal causes a rebound increase in proliferation [125–127]. In contrast, cocaine has no effect on granule cell precursor proliferation in either the dentate gyrus or the SVZ [118].

7.3.8 Neuropeptides and Growth Factors

Several neuropeptides and growth factors have been reported to regulate neuronal precursor proliferation in the adult brain. The peptide neurotransmitter pituitary adenylate cyclase-activating peptide (PACAP), when infused intracerebroventricularly (i.c.v.) for three to seven days, increases cell proliferation both in the SVZ and dentate gyrus of adult mice [128]. The high-affinity PACAP receptor PAC1 is expressed in both the SVZ and the dentate gyrus granule cells layer [128], but it is not known whether this receptor is expressed by precursor cells within these regions. Infusion of sonic hedgehog, a soluble signaling molecule critical for early central nervous system (CNS) development, increases neurogenesis in the adult SVZ and dentate gyrus [129, 130]. In the dentate gyrus, it is unclear whether the effect is on proliferation or survival, but in the SVZ the effect is on proliferation. VEGF also increases neurogenesis in the olfactory bulb and dentate gyrus [131]; it is uncertain whether the effect was on proliferation or survival, although survival seems more likely. Heparin binding epidermal growth factor (HB-EGF) i.c.v. infusion for three days increases neurogenesis in the adult SVZ and dentate gyrus [132, 133]. This effect can most likely be attributed to a change in proliferation, since BrdU was given simultaneously with treatment for only three days. HB-EGF has the potential to regulate proliferation directly, because the EGF receptor has been found on dividing cells in the dentate gyrus [134].

Multiple studies have shown that IGF increases adult neurogenesis in the dentate gyrus [135, 136]. In these studies, BrdU was given several days before sacrifice and treatment continued during the entire period after BrdU labeling, so it is not clear whether the effect of IGF is on proliferation or cell survival. However, longer time points after BrdU, with continued IGF treatment, did not result in larger increases,

suggesting that this effect may primarily influence cell proliferation. Interestingly, one study showed that adult neurogenesis in the dentate gyrus increases with peripheral infusion of IGF [135], suggesting that endogenous IGF produced in the liver may normally regulate cell proliferation in the dentate gyrus. Anti-IGF does not inhibit cell proliferation in the dentate gyrus of mice in standard laboratory housing, but subcutaneous infusion of this antibody prevents running-induced increase in granule cell precursor proliferation (see Section 7.3.1 above) [64]. IGF receptors have been found in the dentate gyrus, but it is not clear whether or not they are expressed by granule cell precursors specifically.

7.4 CONCLUSIONS

Specific types of experiences regulate adult neurogenesis using hormones, neurotransmitters, and growth factors as mediators. A large number of regulatory factors have now been identified, and although no coherent picture has emerged to connect them, it has become clear that distinct factors control proliferation and neuronal survival. Survival of young granule cells in both the dentate gyrus and olfactory bulb appears to be increased by functionally relevant input. Environmental enrichment, that is, increasing exposure to stimuli, increases young granule cell survival in both regions, but the effective stimuli are system specific. For example, the strongest stimulus for altering survival of new neurons in the dentate gyrus appears to be hippocampus-dependent learning, while the best stimulus for survival of new olfactory bulb granule neurons is a variety of odors. Activation and other regulatory factors could potentially act directly on the new neurons to influence their survival. While the age at which young granule cells make synapses is not known, they do receive synaptic input while still at a morphologically immature stage [137–140] and express glucocorticoid receptors very soon after their birth.

Only a few factors regulating the proliferation of olfactory bulb granule cell precursors have been identified. This may reflect the large distance between the site of proliferation in the SVZ and the final location of the new neurons in the olfactory bulb. In the dentate gyrus, where granule cell precursors reside very close to the mature granule cells, a large number of proliferative and antiproliferative factors are now known. One feature that many of these regulators have in common is that they have parallel effects on both cell proliferation and depressive symptoms. Factors that increase granule cell precursor proliferation have antidepressant properties, either in humans or animal models, while factors that inhibit proliferation are correlated with depressive symptoms. For example, stress and glucocorticoids dramatically inhibit granule cell precursor proliferation as well as survival, and are strongly linked with depression [141–143]. In addition, cell proliferation in the dentate gyrus is increased by the classic SSRI antidepressants as well as all other classes of commonly used antidepressants that have been tested, including tricyclic antidepressants, monoamine oxidase inhibitors, specific norepinephrine reuptake inhibitors, phosphodiesterase-IV inhibitors, and electroconvulsive seizure therapy. Although not yet in clinical use, antagonists of NMDA and metabotropic subtypes of glutamate receptors, both of which increase cell proliferation in the dentate gyrus, have also been found to have antidepressant properties. Interestingly, this relationship also holds for the AMPA receptor positive modulators, which have both proliferative and

antidepressant properties, despite having an effect that is opposite of the NMDA receptor antagonists at the cellular level; that is, they are excitatory rather than inhibitory. In contrast, antipsychotic medications and cocaine have no impact on adult neurogenesis or on depressive symptoms. While a link between adult neurogenesis in the dentate gyrus and depressive illness has yet to be proven, there does appear to be a strong correlation between factors that relieve depressive symptoms and those that increase adult neurogenesis in the dentate gyrus.

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8

NEUROTROPHIC FACTORS

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8.1	Introduction	221
8.2	Physiological Functions of NTFs	222
8.2.1	Neuronal Survival and Differentiation During Development	222
8.2.2	Neurogenesis in the Adult	224
8.2.3	Neuronal Maturation During Development	224
8.2.4	Modulation of Neuronal Functions in the Adult	224
8.3	Therapeutic Applications	225
8.3.1	Amyotrophic Lateral Sclerosis	225
8.3.2	Peripheral Neuropathies	227
8.3.3	Alzheimer's Disease and Cognitive Impairment	227
8.3.4	Parkinson's Disease	228
8.3.5	Pain	229
8.3.6	Other Indications	229
8.4	Drug Discovery based on NTF Mechanisms	230
8.4.1	Neurotrophic Factors as Drugs	230
8.4.2	Biological Antagonists	231
8.4.3	Direct Receptor Agonists and Antagonists	231
8.4.4	Indirect Modulators of NTF Function	232
8.5	Conclusions	232
	References	233

8.1 INTRODUCTION

The discovery of nerve growth factor (NGF) half a century ago initiated the investigation of the field of neurotrophic factors (NTFs). The first recognized function was as survival factors for neurons during certain stages of development. Limited availability of a specific NTF determined neuronal survival, matching the number of neurons to the requirements of the receptive tissue. NGF was the first identified member of the neurotrophins, a family of proteins which include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). A second family of NTFs consists of the glial cell-derived neurotrophic

factor (GDNF) and the related proteins neurturin, artemin, and persephin. Additionally, members of the large family of transforming growth factor- β (TGF β) and other groups of secreted proteins have been discovered which serve similar roles as the neurotrophins. The recognized functions of these proteins gradually enlarged beyond the constraints of the original NTF concepts. In addition to regulating neuronal survival, NTFs are now known to regulate differentiation and maturation of a variety of types of neurons. Although the requirement for NTFs for survival appears to be confined to certain developmental stages, the modulation of structure and expression of differentiated function continues throughout the life span. In addition, NTFs have acute, neurotransmitter-like functions in the adult nervous system. Despite more than half a century of research, the full functional role of NTFs continues to evolve.

8.2 PHYSIOLOGICAL FUNCTIONS OF NTFS

This chapter will focus on the two dominant protein families of NTFs, the neurotrophins and the GDNF-related proteins. Figures 8.1 and 8.2 show these proteins and their corresponding receptor interactions. Individual factors and their receptors participate in a wide array of physiological functions.

8.2.1 Neuronal Survival and Differentiation During Development

Many studies have confirmed the original concept that NTFs are regulators of cell survival during early development. There can be, in some circumstances, a high

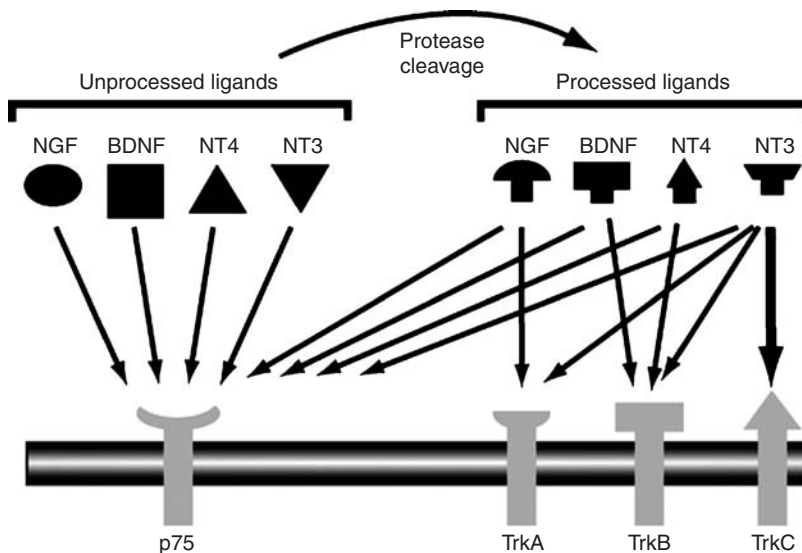


Figure 8.1 Neurotrophins and their receptors. NGF, BDNF, NT-3, and neurotrophin-4/5 (NT-4) bind selectively to corresponding Trk receptors (right side of graph). The Trk tyrosine kinase receptors stimulate multiple signal transduction pathways. All neurotrophins and their unprocessed precursors bind to the p75 neurotrophin receptor (p75NTR, p75). The p75NTR interacts with several other proteins that regulate intracellular functions. (Reprinted with permission from [47].)

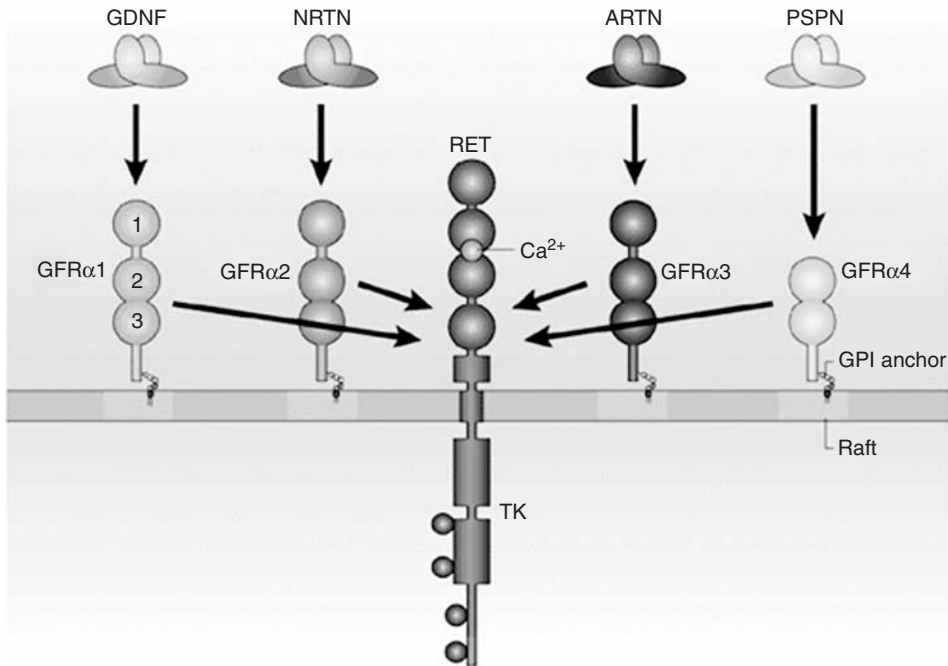


Figure 8.2 GDNF-related proteins and their receptors. GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) bind selectively to their corresponding GDNF family receptor- α (GFR α) subtype, which interacts with the RET tyrosine kinase to stimulate signaling. (Reprinted with permission from [48].)

degree of specificity linking a specific neuronal population with a specific neurotrophin and a specific tissue. The most elegant and best understood example is that of sensory neurons which differentiate into highly specific subpopulations with specialized terminations as nociceptive cutaneous free endings or on Merkel cell mechanoreceptors, muscle spindles, and hair follicles. Each of these subpopulations depends on and responds to a specific member of the neurotrophin family, which are respectively NGF, BDNF, NT-3, and NT-4/5 [1, 2]. Studies with antineurotrophin antibodies and of mice with null mutations of the various neurotrophins or their respective Trk tyrosine kinase receptors confirm this well-established picture for the sensory neurons.

Other neuronal populations dependent on or responsive to members of the neurotrophin family include sympathetic neurons (NGF), cholinergic basal forebrain neurons (NGF), spinal cord motoneurons (BDNF), hippocampal pyramidal neurons (BDNF), and retinal ganglion cells (BDNF, NT-3). Beyond the neurotrophin family, several members of the GDNF protein family have been characterized as regulators of neuronal survival during early development. Enteric and motor neurons depend for their early development survival on individual members of the GDNF protein family, with different specificities [3]. Similar roles have been established for TGF β family members [4].

Although the primary focus of initial research was on promotion of survival due to NTFs, neurotrophins can also induce apoptosis. Both the receptor mechanisms

and biology create a complex picture. Under conditions of reduced or absent Trk signaling, a different receptor for neurotrophins, the p75 neurotrophin receptor (p75NTR), can promote apoptosis following ligand binding. For this to occur, p75NTR forms a receptor complex with sortilin, a transmembrane protein with a very short cytoplasmic domain [5]. Furthermore, the processing of and state of the neurotrophin can affect the balance between promoting survival or death. For example, the effect of NGF on cell survival depends on proteolytic processing. Unprocessed proNGF has a fivefold greater affinity for p75NTR than mature NGF and has negligible binding to the TrkA tyrosine kinase receptor, making proNGF a more potent inducer of cell death than mature NGF [6].

8.2.2 Neurogenesis in the Adult

Neurogenesis is not confined to development but continues into adulthood, and NTFs continue to play a role in the regulation of neurogenesis in the adult nervous system. Among the neurotrophins, BDNF and NT-3 appear to be involved in the regulation of neurogenesis in the cortical ventricular and subventricular zones from which the newly generated neurons emanate to cortical areas. Cell culture studies suggest that individual neurotrophins regulate survival and differentiation of progenitor cells by distinct pathways. BDNF has been recognized as a key regulator of neurogenesis in the hippocampus [7].

8.2.3 Neuronal Maturation During Development

Neurotrophic factors promote growth of dendrites and axons as well as the molecular differentiation of many populations of neurons. They stimulate the extension of the neurites and provide cues for targeting to the appropriate post-synaptic cells. Among the best studied examples is the role of NGF in the differentiation of sympathetic neurons of the peripheral nervous system. Cell culture and in vivo studies revealed that NGF promotes dendritic and axonal growth, acts as a chemoattractant, and stimulates the expression of genes encoding proteins necessary for synaptic functions [8]. All members of the neurotrophin and GDNF-related protein families participate in growth and targeting of sensory and motor neurons of the peripheral nervous system. In the central nervous system (CNS), dendritic morphology of pyramidal cells is influenced by BDNF, and the cholinergic neurons of the basal forebrain respond to NGF, BDNF, and NT-3 in specific ways. The examples suggest that the NTFs serve as promoters and regulators of morphological and molecular maturation, with a high degree of molecular and cellular specificity. They interface with many other proteins that determine developmental growth and targeting [9].

8.2.4 Modulation of Neuronal Functions in the Adult

In addition to their roles in the generation and differentiation of neurons, some of the neurotrophic proteins have been recognized, surprisingly, to have a role as signaling molecules, with actions that occur over much shorter time spans than the better known morphological effects. In the brain, the synthesis of BDNF is regulated by synaptic activity, and the protein is released during synaptic activity. BDNF

contributes to the strengthening of the synaptic signal over multiple neurotransmitter discharges, which are believed to underlie memory functions. Mice with null mutations of the BDNF gene have deficits in the formation of long-term potentiation (LTP) and in behavioral aspects of memory [10].

A parallel role for NGF has been discovered in the function of the peripheral sensory nervous system. Pain-mediating A δ and C fibers express TrkA receptors. Injections of NGF into normal skin causes prolonged pain sensation. Tissue injury and inflammation strongly upregulate the synthesis and release of NGF from keratinocytes, fibroblasts, and muscle cells. NGF stimulates pain transmission by the sensory neurons through direct and indirect mechanisms [11]. The TrkA tyrosine kinase receptors activate multiple target proteins within the synaptic terminal. Local effects of NGF include sensitization of the temperature-sensitive TRPV1 channels and voltage-dependent ion channels. While not as rapid as the synaptic effects of BDNF, the NGF effects on nociceptive neurons directly influence neuronal activity in morphologically mature neurons.

8.3 THERAPEUTIC APPLICATIONS

The various physiological roles of the NTFs offer many obvious possibilities to use the proteins and their receptor mechanism for drug discovery. In the early attempts the proteins themselves were administered systemically or intrathecally for degenerative diseases of the peripheral or central nervous systems, respectively [12, 13]. These efforts did not lead to successful, approved drugs. More recently, the efforts have shifted to alternative administration attempts, including local injection of protein factors or gene therapy. Furthermore, the detailed understanding of NTFs and receptor mechanisms has opened new possibilities to generate drugs that mimic or antagonize neurotrophin functions and allow for the therapeutic intervention in diseases in which they are involved [14]. The following section focuses on diseases and conditions with strong rationale for therapeutic interventions based on NTF mechanisms (Table 8.1). For many of them, early clinical experiences have been obtained which help to guide the currently ongoing drug discovery and development efforts.

8.3.1 Amyotrophic Lateral Sclerosis

In amyotrophic lateral sclerosis (ALS) the primary motor neurons that innervate and control skeletal muscles degenerate over a period of a few years. The upper, cortico spinal neurons as well as the lower, spinomuscular motor neurons are affected, causing spasticity, hyperreflexia, and muscle atrophy and finally death because of respiratory failure. Environmental and genetic causes have been identified. A small number of familial forms carry mutations in the gene for Cu/Zn superoxide dismutase (SOD). This familial form shares the selective motor neuron degeneration and the other pathological features of ALS. It is possible that the mutations in SOD, an enzyme that contains Cu²⁺ and catalyzes the conversion of superoxide radicals to hydrogen peroxide and oxygen, cause the enzyme to generate alternative, more toxic oxidative species or may release the toxic copper ion. The oxidative species may cause the aggregation of neurofilaments that are seen in cell bodies and axons of motor neurons in ALS patients and of transgenic mice with SOD disease genes.

TABLE 8.1 Therapeutic Applications of NTF-Related Drugs

Indication	Neurotrophic Factors	Therapeutic Agent and Delivery
Amyotrophic lateral sclerosis	BDNF, NT-3, CNTF, IGF-1	Systemic or local administration of protein or agonists, gene therapy
Peripheral neuropathies	NGF, NT-3, GDNF	Systemic administration of protein or agonists
Alzheimer's disease	NGF	Intracerebral infusions or gene therapy delivery to cholinergic neurons in basal forebrain
Parkinson's disease	GDNF, neurturin	Intracerebral infusion or gene therapy delivery to basal ganglia
Stroke and acute injury	BDNF, NT-3, TGF β	Systemic or local administration
Depression	BDNF	Brain-penetrant mimetics
Pain	Anti-NGF	NGF-capturing agents or antagonists

Abbreviations: CNTF, ciliary neurotrophic factor; IGF-1, insulin-like growth factor type 1.

ALS was the first testing ground for neurotrophic therapy, shortly after the time of discovery of the neurotrophin family and the associated receptors. Several growth factor proteins, including BDNF, CNTF, and IGF-1, delayed the progression of degenerative events in developmental animal models of motor neuron degeneration. Although some evidence of activity was obtained in a human trial with IGF-1, overall, extensive clinical studies with systemic administration of these proteins failed to provide compelling evidence for efficacy in ALS. BDNF was also administered intrathecally, without success. In retrospect, one important issue may have been that the animal model data suggesting beneficial effects on motor neuron survival was obtained in early development, rather than in progressive degeneration of established motor neurons in the adult. Additionally, in these early clinical trials, little attention was paid to pharmacokinetics. Whether the amounts of the growth factors administered systemically were adequate to achieve adequate levels of receptor occupancy and activation on the targeted neurons was not established.

More recent efforts attempt to address these questions and to provide adequate stimulation of the neurotrophin receptor mechanisms. BDNF has been of particular interest since motor neurons express TrkB receptors, and TrkB knockout (KO) animals show motor neuron loss. Studies with BDNF are underway in which the protein is infused into the ventricular system in an attempt to better reach the upper motor neurons. Biochemical and imaging surrogate markers of motor neuron functions are being employed to monitor efficacy, in addition to the behavioral clinical analysis. Nevertheless, the initial clinical studies have not produced encouraging results [15]. Recent studies in an improved animal model of ALS, transgenic mice animals carrying the SOD mutation, have provided compelling evidence that a gene therapy strategy might be more successful. In the animal studies, GDNF was

generated in muscular tissue by administration of an adeno-associated virus vector. The treatment prolonged the life span of the animals [16]. These positive effects in a highly relevant animal model of ALS renew the enthusiasm for NTF therapy. A combination of strategies that provide beneficial effects for upper and lower motor neurons is expected to prolong the life span of people who suffer from this devastating disease [17].

8.3.2 Peripheral Neuropathies

Neuropathy can be caused either by demyelination or retrograde degeneration of the axonal terminals or a combination of both processes. Sensory neuropathy is most frequent and is manifested by numbness, abnormal sensations, and pain. Autonomic neuropathy can occur as an isolated syndrome or in accompaniment with sensory neuropathy and leads to dysfunction in cardiovascular and visceral functions. Neuropathy can be caused by metabolic disturbances such as diabetes, inflammation, toxins, or drug, particularly certain cancer chemotherapies. A significant proportion of neuropathies are hereditary. The most common of the hereditary motor and sensory neuropathies, also known as Charcot–Marie–Tooth disease, exist in a number of variants. Varying degrees of progressive demyelination and axonal atrophy underlie these hereditary neuropathies. The neurons with the longest axons are typically most strongly affected. Specific mutations in peripheral myelin protein 22, myelin protein P0, or connexin 22 have been identified in some familial cohorts.

Neurotrophic factors may provide a general approach to the therapy of neuropathy, since the growth factors stimulate the synthesis of proteins involved in signaling functions and regenerative growth [18]. In cell culture and animal models of neuropathies they increase the resistance of neurons to various toxins and to procedures that replicate aspects of the disease process. NGF targets small nociceptive sensory neurons and sympathetic neurons. Evidence of efficacy was seen with NGF in animal models of diabetic or chemotherapy neuropathy. Because of the specificity for nociceptors, NGF was assessed in clinical trials for the treatment of painful diabetic neuropathy. However, extensive clinical studies failed to demonstrate significant efficacy [19]. It may be noteworthy that dose levels of NGF were restricted in human subjects due to a frequent side effect of painful allodynia and hypersensitivity. Dose levels in the human trials were around 1000-fold lower than in the animal models.

The receptors for NT-3, TrkC, are preferentially expressed by the subpopulation of proprioceptive sensory neurons which innervate muscle spindles. This subpopulation of sensory neurons is particularly sensitive to anticancer drugs. Positive animal model studies support the potential for clinical development [20]. Recent studies also support the use of gene therapy. A herpes simplex virus–based vector of NGF and NT-3 was reported to be effective in an animal model of cisplatin-induced neuropathy [21]. Most sensory neurons express receptors for members of the GDNF protein family, providing an alternative to therapy with neurotrophins. Similar to the situation in ALS, local production of the NTFs by gene therapy may be effective for therapeutic purposes.

8.3.3 Alzheimer's Disease and Cognitive Impairment

The formation of amyloid plaques and neurofibrillary tangles is believed to be the primary pathological event in Alzheimer's disease, which eventually leads to

neuronal degeneration. Early in the course of the disease, the cholinergic neurons of the basal forebrain undergo atrophic changes, which decreases their regulatory input to hippocampal and cortical structures relevant for memory functions. The cholinergic neurons of the basal forebrain selectively express TrkA receptors for NGF, suggesting that administration of NGF into the brain may counteract the cholinergic neuron degeneration in Alzheimer's disease and so restore some of the dependent memory functions [22]. Limited clinical trials in which NGF was infused into the cerebral ventricles of Alzheimer's patients were conducted without success. Subsequent animal studies showed that when NGF is given intraventricularly, most of it travels with cerebrospinal (CSF) flow to the spinal canal, where it may stimulate the TrkA-expressing sensory neurons that mediate pain. It is thus necessary to provide a local source of NGF to the cholinergic neurons. Delivery of NGF to the basal forebrain by cell therapy or gene therapy has been successfully performed in animal models. Preliminary clinical studies in which NGF is delivered to the basal forebrain by cell therapy or gene therapy have been reported [23].

The involvement of BDNF in memory functions has led to speculation that BDNF and TrkB may be good targets for drugs which directly influence memory processes [24]. Particularly potentiation of BDNF actions may enhance the performance of the systems that underlie the formation and retrieval of memories. Cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) activation, a central step in memory functions, stimulates BDNF synthesis. Since the protein NTFs do not pass the blood-brain barrier, it will be necessary to identify local means of delivery such as cell or gene therapies, small-molecule activators, or potentiators. No suitable compounds have been identified as yet, but the conceptual attraction of memory-enhancing drugs is likely to keep these efforts alive.

8.3.4 Parkinson's Disease

Neurotrophic factor therapy seems uniquely attractive for Parkinson's disease, since much of the pathophysiology is related to degeneration of a single neuronal population, the dopaminergic neurons of the substantia nigra. In animal models, several NTFs were shown to increase the resistance of dopaminergic neurons to toxic insults and to increase their capacity for compensatory adaptations. In the neurotrophin family, BDNF and NT-4/5, both of which activate the TrkB tyrosine kinase receptors, have mild beneficial effects in animals with experimentally induced degeneration of the dopaminergic cells. Very robust effects were obtained with GDNF and neurturin. In an early clinical trial, GDNF was infused into the ventricles of Parkinson's patients through chronically implanted shunts and infusion pumps. Because of various adverse effects and the absence of convincing positive signals, in further trials GDNF was infused directly into the target areas of dopaminergic neurons. First trials provided tentative evidence for clinical efficacy which, however, proved difficult to substantiate in further double-blind studies [25, 26]. Similar to the situation in ALS and Alzheimer's disease, delivery of the factors by gene therapy rather than local infusion may produce more robust results.

The positive results with protein growth factors in animal models of Parkinson's disease and the difficulty with delivery to the CNS in humans prompted efforts to identify small-molecule, direct or indirect, mimetics. Neurotrophic factors activate several pathways, including the stress pathways and the c-Jun N-terminal kinase

(JNK). Inhibitors of JNK and of kinases upstream of JNK are effective in animal models of Parkinson's disease, including CEP-1347, which has been taken into clinical development [27]. During development, the NTFs regulate apoptotic degeneration of neurons, and it has been speculated that apoptosis may contribute to the degeneration of dopaminergic neurons in Parkinson's disease. Inhibitors of caspases, the proteases catalyzing protein degradation during apoptosis, are effective in cell culture models of Parkinson's disease [28]. These approaches are intriguing examples for the view that regulators of downstream events of neurotrophic receptors may lead to therapeutically useful small-molecule mimetics.

8.3.5 Pain

NGF plays an important role as a survival factor for nociceptive sensory neurons during development and as a modulator of differentiated function in the adult. Upregulation of NGF levels occurs as part of the tissue response to injury or inflammation. NGF activates TrkA receptors on sensory neurons and modifies the function of TRPV1 channels and axonal sodium channels to enhance pain signal transmission. The released NGF also stimulates TrkA receptors located on mast cells, which then release several other pain mediators to further enhance the pain response. The stimulation of TrkA receptors by NGF on sensory neurons results in retrograde signaling to the cell body of the sensory neurons and enhanced expression of genes that encode precursors of neuropeptide transmitters which contribute to the long-term sensitization of the pain response. Administration of NGF to animals or humans results in long-lasting painful allodynia and hypersensitivity. Antagonists against NGF are effective in animal models of acute and chronic pain [29]. Inhibitory proteins, including peptibodies and antibodies against NGF, are being pursued as analgesic therapies in human clinical trials. Other biological and small-molecule antagonists to NGF receptors could also be candidates for clinical investigation. In addition to NGF, BDNF and GDNF may participate in the primary pain response and provide an avenue to the discovery of new drugs for this indication [30].

8.3.6 Other Indications

Neurotrophic factors and their mechanisms are likely to offer avenues for successful drug discovery for other indications beyond those discussed above. One potential area of interest is depression, which may be related to BDNF. Depression is associated with a decline of neurogenesis in the brain, a process in part regulated by this neurotrophin. Brain-penetrant BDNF mimetics may exhibit antidepressant activity in patients ([31]; also see Chapters 19 and 20 in Volume I of this hand book.).

Obesity and weight control are influenced by several NTFs. CNTF reduced the weight of human patients in a clinical trial for ALS and is now being pursued for obesity [32]. TrkB receptors mediating the actions of BDNF and NT-4/5 are located in parts of the brain, including the hypothalamus, important for the control of food intake and metabolism [33]. The expression of several neurotrophins and growth factors from other protein families is upregulated following ischemic stroke and acute injury to the brain. Potentially growth factors may play an important role in recovery from these acute insults and in drug discovery efforts at enhancing this process [34]. Table 8.1 lists the currently known possibilities for drug discovery based

TABLE 8.2 Types of Drugs That Stimulate or Inhibit NTF Actions

Agonistic Agents	Antagonistic Agents
Native NTFs	
Modified NTFs (e.g., pegylated proteins)	
Agonistic peptide fragments	Antagonistic peptide fragments
	Peptibodies: peptides binding to NTFs and linked to immunoglobulin G (IgG) fragment
	Neutralizing antibodies
	Soluble receptor–IgG fusion proteins
Agonistic ligands to NTF binding site	Antagonistic ligands to NTF binding site
	Inhibitors of receptor Trk function
Activators of downstream mediators	Inhibitors of downstream mediators

on NTF mechanisms. They reflect the complex patterns of biological activity these proteins display in the nervous system. Many as-yet-undiscovered possibilities will likely cause this list to grow in the future.

8.4 DRUG DISCOVERY BASED ON NTF MECHANISMS

The NTFs, their receptors, and the downstream signaling cascades offer several putative drug targets and intervention points for pharmacological manipulation (Table 8.2). Agonistic effects are desired in most situations—for example GDNF in Parkinson’s disease and BDNF in depression. Antagonistic effects are desired to counter the role of NGF in pain. Current drug discovery and development efforts use a broad spectrum of biologics and small-molecule drug candidates.

8.4.1 Neurotrophic Factors as Drugs

Several natural proteins, for example, insulin and growth hormone, are medically used as effective drugs. Systemic injections of these proteins achieve adequate plasma levels and receptor occupancy to elicit the desired biological effects. Detectable plasma levels were achieved with systemic administration of NTFs taken in clinical trials . As expected for proteins, their half-lives were in the range of a several hours. It may be possible to increase their half-lives by introducing chemical modifications, such as pegylation, a method successfully used to prolong the half-lives of other therapeutic proteins [35]. Prolongation of half-life in turn could lead to prolongation of biological response.

The use of NTFs for diseases of the CNS faces a special hurdle, since the proteins do not pass the blood-brain barrier. Several methods of intracerebral administration have been attempted. In initial clinical trials, some of the factors were infused into the ventricles of patients through chronically implanted shunts and infusion pumps. These efforts have been largely abandoned because, rather than penetrating into the brain parenchyma to reach the desired target, most of the infused protein is rapidly transported away by CSF flow to the spinal canal. An alternative strategy has been

direct infusion of the NTF into brain tissue. Various technical and medical hurdles limit the utility of these infusion strategies [36]. Gene therapy, while still requiring stereotaxic surgery for local implantation of the vector to the brain, appears to be a promising alternative. While still at early experimental stages, gene therapy technologies offer the prospect to achieve long-term expression of NTFs at the targeted brain area [37]. Cell therapy, implantation of cells genetically manipulated to overexpress proteins of interest, is an interesting approach which uses the strength of gene therapy but avoids potential issues with use of viral vectors in humans.

8.4.2 Biological Antagonists

Several attractive strategies are available to generate biological therapeutic antagonists to proteins. Antibodies have shown their utility as drugs in the immunology and cancer field. They are highly selective and potent and have attractive pharmacokinetic properties. A monoclonal anti-NGF antibody currently under development for pain management is the first NTF antibody drug candidate in clinical trials. Advanced technologies make it possible to generate humanized or human monoclonal antibodies, with little potential for adverse immune reactions. The long half-life of antibodies allows design of therapeutics which require administration at intervals of one month or more.

As an alternative to antibodies, soluble receptors have been produced as capturing agents and antagonists for several proteins. In the neurotrophic factor field, a TrkA–IgG fusion protein was generated by fusing the extracellular domain of TrkA to part of a human immunoglobulin [38]. This molecule binds NGF with high affinity and is effective in animal models of inflammatory pain [29]. Fusion proteins have a higher risk than humanized antibodies to provoke an immune response in humans because of the neoepitopes generated at the interface of the two parts. Peptibodies are a further innovative approach to produce NTF antagonists. In these molecules, peptides occupying the binding sites necessary for the interactions of the receptors are fused to and metabolically stabilized by the antibody domains. An anti-NGF peptibody was shown to be effective in animal models of pain [39]. As the other fusion proteins, peptibodies carry substantial risk of an immune response with chronic therapeutic use.

8.4.3 Direct Receptor Agonists and Antagonists

Binding of NTFs to their receptors involves several binding sites. The active receptor complexes typically involve several proteins. These complex, multifaceted interactions may explain the limited success so far in the search for direct NTF mimetics. Conceptually, it seems easier to generate antagonists, since blocking of a single site of interaction might be sufficient to disturb the receptor function. The interaction between neurotrophins and their Trk receptors involves two domains, or “patches”. The specificity patch defines the selective interaction of the neurotrophins with their respective Trk receptors and is formed by the N-terminal amino acids which bind to the top of the receptor [40]. Compounds binding with high affinity to this domain should be able to selectively block the activation of specific Trk receptors. The second, conserved patch receives contributions from both monomers of the neurotrophins and is formed by amino acids highly conserved in the neurotrophin family,

making it less attractive as a receptor site for selective Trk inhibitors. Two compounds have been reported which demonstrate the feasibility of finding direct NTF receptor antagonists. ALE-0540 is an inhibitor of NGF to TrkA and p75NTR with affinities around 5 μ M. It is effective in animal models of pain when given by intrathecal injections [41]. PD90780 inhibits NGF binding to p75NTR with an affinity of 3 μ M without inhibiting the binding of BDNF or NT-3 [42].

Rather than inhibiting binding of NTFs to their receptors, it is possible to interfere with the effector function of the receptor. Tyrosine kinase receptors have been recognized as attractive drug targets in the cancer field, where several drugs and drug candidates inhibit the function of tyrosine kinase growth factor receptors. Most of the known inhibitors block the binding of adenosine triphosphate (ATP) to the catalytic tyrosine kinase domain on the cytoplasmic side of the receptors. For the neurotrophin receptors, the compound K252a has been identified as a potent inhibitor of Trk [43]. It is effective in an animal model of pain [44]. It lacks adequate selectivity for Trk receptors and is likely to produce multiple adverse effects reflecting the binding to other tyrosine kinase receptors but demonstrates the feasibility of the approach.

8.4.4 Indirect Modulators of NTF Function

Events downstream of the NTF receptors provide alternative putative drug targets that appear to have higher technical feasibility of success than the NTFs themselves. Neurotrophic factors activate several overlapping pathways, including mitogen-activated protein (MAP) kinase, phosphatidylinositol-3-kinase, and phospholipase-C- γ pathways. The compound CEP-1347, an inhibitor of JNK and MLK, is being pursued for Parkinson's disease [27]. Other drug discovery approaches related to NTFs include attempts to increase their synthesis by modifying immunophilins, proteins that have been linked to axonal regeneration in the nervous system. Ligands for immunophilins have been taken into clinical trials for Parkinson's disease based on results in animal models of Parkinson's disease [45]. Ampakines, potentiators of the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptors, have been shown to enhance the synthesis of BDNF. These compounds promote the survival of dopaminergic neurons in animal models of Parkinson's disease [46]. These initial data demonstrate that it will be possible to find small, brain-penetrant molecules able to cross the blood-brain barrier and to stimulate NTF mechanisms in the brain.

8.5 CONCLUSIONS

The neurotrophin and GDNF-related protein families are the principal members of the NTF group, though several other trophic factors or cytokines also display neurotrophic activity and are often included in this group. NTFs were initially characterized as regulators of neuronal growth and differentiation during development. The role of NTFs in the adult has more recently been recognized to be that of modulators of differentiated functions such as neurotransmitter production or even to function themselves directly at synapses. Neurotrophic factors have been pursued for several diseases characterized by neurodegeneration, including ALS, Alzheimer's

disease, Parkinson's disease, and diabetic peripheral neuropathy. Although studies continue in degenerative diseases, recent studies suggest potential functional roles for NTFs in pain, memory, depression, and weight control. Antagonists to neurotrophins, NGF in particular, are under study as analgesic agents. Enhancers of the effects of BDNF appear promising for the treatment of depression. For the agonistic use of NTFs, modified proteins, local infusion, cellular therapy, and gene therapy delivery approaches are being attempted. Small-molecule mimetics may provide the best means for circumventing the blood–brain barrier to proteins. For antagonists, antibodies, receptor fusion proteins, peptibodies, as well as direct and indirect small-molecule receptor antagonists are being developed. The emerging pharmacology of NTFs will hopefully lead to effective drugs for a broad array of human diseases.

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9

NEUROTROPHINS AND THEIR RECEPTORS

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9.1	Historical Introduction	237
9.1.1	Neurotrophins	237
9.1.2	Neurotrophin Receptors	238
9.2	Biological Functions of Neurotrophin Signaling	238
9.3	Structure of Neurotrophins	239
9.4	Structure of Neurotrophin Receptors	240
9.4.1	Structure of p75 ^{NTR}	240
9.4.2	Structure of Trk Receptors	241
9.5	Signaling by Trk Receptors	242
9.5.1	Determinants of Neurotrophin Preference	242
9.5.2	Signal Transduction Pathways	242
9.5.3	Other Signaling Proteins That Associate with Trk Receptors	243
9.5.4	Influence of Trk Splice Variants on Signaling	245
9.6	p75 ^{NTR} Signaling	246
9.7	Functional Interactions of p75 ^{NTR} and Trk Receptors	247
9.8	Retrograde Axonal Signaling	248
9.9	Summary	249
	References	249

9.1 HISTORICAL INTRODUCTION

9.1.1 Neurotrophins

The discovery and isolation of nerve growth factor (NGF) [1] was a seminal event in the history of developmental neurobiology. Although initial studies focused on the ability of NGF to promote survival and axon growth of sensory and sympathetic neurons of the peripheral nervous system, studies over half a century have revealed that NGF influences many aspects of neural development, in the central nervous system as well as the peripheral nervous system, and have demonstrated important

functions of NGF for a few nonneuronal cell types as well. The observation that sensory neurons of some cranial ganglia were not responsive to NGF but were dependent on an NGF-like factor in the tissues they innervate led to the discovery and isolation of brain-derived neurotrophic factor (BDNF), which was found to have an amino acid sequence that was substantially similar to that of NGF [2]. This similarity immediately led several groups to employ degenerate polymerase chain reaction (PCR) primers, based on the nucleotide sequences of NGF and BDNF, to clone additional structurally related proteins: neurotrophin-3 (NT-3) [3–5] and neurotrophin-4 (NT-4; also sometimes referred to as NT-4/5) [6–8]. These four proteins, collectively known as neurotrophins, are critically important for the development and adult function of the mammalian nervous system and for the development of some nonneural tissues, as will be discussed at length in this chapter. Neurotrophins, varying in number, exist in nonmammalian vertebrates also, but this will not be discussed here.

9.1.2 Neurotrophin Receptors

The existence of specific neurotrophin receptors was first revealed by the discovery of specific high-affinity cell surface binding of radio-iodinated NGF. Binding studies revealed heterogeneity of affinity of these binding sites [9–11]. The cause of this heterogeneity was explained, at least in part, when molecular cloning of the receptors was achieved. The actions of the neurotrophins are mediated by the protein products of four different neurotrophin receptor genes. These receptors possess varying degrees of selectivity for particular neurotrophins. The first neurotrophin receptor to be cloned, the 75-kDa neurotrophin receptor ($p75^{\text{NTR}}$) [12–14] binds all four neurotrophins [15, 16]. It quickly became clear, however, that this receptor was not sufficient to mediate many of the known cellular responses to neurotrophins. Unlike the receptors for many other growth factors, $p75^{\text{NTR}}$ is not a receptor tyrosine kinase, yet biochemical studies suggested the existence of an NGF-activated receptor tyrosine kinase [17–19]. This recognition coupled with the observation that the normal cellular counterpart of an oncogenic tyrosine kinase created by a gene fusion event (tropomyosin-related kinase, Trk) is highly expressed in known neuronal targets of NGF action [20, 21] led to the discovery that the Trk proto-oncogene is an NGF receptor [22–24]. Earlier studies had revealed that a homolog of Trk, TrkB, also is expressed primarily in the nervous system [25, 26]. It came as no surprise, therefore, that TrkB also proved to be a neurotrophin receptor the tyrosine kinase activity of which was activated by BDNF and NT-4 and less effectively by NT-3 [8, 27–29]. Furthermore, another Trk homolog, TrkC, was identified and shown to be a receptor for NT-3 [30]. For the sake of consistency, it has become conventional to refer to Trk as “TrkA” and to employ the term “Trk” to refer jointly to the three structurally related receptors. $p75^{\text{NTR}}$ and the three Trk receptors jointly represent a receptor system of exceptional complexity, because, as will be discussed in detail in later sections, $p75^{\text{NTR}}$ and the Trk receptors can signal independently, but when coexpressed, $p75^{\text{NTR}}$ influences the signaling of Trk receptors, and Trk receptors influence the signaling of $p75^{\text{NTR}}$.

9.2 BIOLOGICAL FUNCTIONS OF NEUROTROPHIN SIGNALING

Neurotrophins have a wide range of critical functions in the developing and mature nervous system and have several important functions in nonneural tissues also. A full

discussion of these functions is beyond the scope of this chapter, but it is useful here to briefly illustrate the range of these functions. Neurotrophins were originally discovered in the context of the so-called neurotrophic hypothesis, which states that the survival of developing neurons requires prosurvival factors that the neurons receive from the target they innervate. This certainly is an important function of neurotrophins, particularly in the peripheral nervous system where, for example, survival of sympathetic neurons requires NGF produced by targets of sympathetic innervation and survival of different functional categories of sensory neurons requires one or more neurotrophins, dictated by the particular Trk receptor that is expressed. Remarkably, while neurotrophin signaling through Trk receptors generally promotes neuronal survival, neurotrophin signaling through $p75^{\text{NTR}}$ may promote neuronal apoptotic cell death [31].

Neurotrophins also function widely in promoting neuronal differentiation and in promoting growth of axons and dendrites. Here again, $p75^{\text{NTR}}$ and Trk receptors sometimes function oppositely. For example, while Trk-dependent signaling promotes axon growth and arborization in sympathetic and sensory neurons, $p75$ -dependent signaling suppresses this growth and arborization [32].

Neurotrophins regulate development of neuronal synapses [33] and regulate synaptic function [34]. BDNF and TrkB have emerged as particularly important regulators of synaptic development and function, but $p75^{\text{NTR}}$ also plays an important role [35]. In this context, neurotrophin function extends into the fundamental mechanisms of learning and memory and into psychiatric disease [36, 37]. An emerging area of intense interest is the function of neurotrophins in the neuroendocrine system. A few examples of such functions include the role of hypothalamic BDNF/TrkB signaling in control of energy balance and body weight [38] and the function of $p75^{\text{NTR}}$ in circadian regulation [39].

Functions of neurotrophins in the nervous system are not restricted to neurons but extend to all classes of glial cells as well, including Schwann cells, astrocytes, oligodendrocytes, and microglia. For example, neurotrophins regulate myelination by both Schwann cells and oligodendrocytes [40] and regulate survival of both cell types [41, 42].

Nonneural functions of neurotrophins have been less studied than neural functions, no doubt for historical reasons, so much remains to be learned about such functions. Nevertheless, it is clear that both $p75^{\text{NTR}}$ and Trk receptors mediate a variety of functions in nonneural tissues, including regulation of cardiovascular development [43, 44], regulation of hair follicle development [45], and modulation of immune and inflammatory function [46], to name only a few examples.

9.3 STRUCTURE OF NEUROTROPHINS

Neurotrophins, like most secreted hormones and growth factors, are synthesized as larger precursor proteins, which are processed, with varying efficiency, by cleavage at a dibasic site (Arg–Arg or Lys–Arg) before secretion. Proneurotrophins exist as noncovalently associated homodimers of 27 to 32-kDa polypeptide chains, while mature neurotrophins exist as homodimers of 13 to 14-kDa chains. Cleavage of proneurotrophins is mediated by furin and related prohormone convertases [47–50]. Although neurotrophin heterodimers have not been reported from natural sources, heterodimers do form when two neurotrophins are coexpressed in artificial expression systems [51, 52].

Early studies on the biochemistry of NGF focused on NGF isolated from the submaxillary salivary glands of male mice, a curiously rich source of NGF in mice but not in most other mammals. NGF can be isolated from this source as a specific high-molecular-weight complex (7S-NGF) consisting of NGF itself (referred to as β -NGF in this context) and two other proteins known as α and γ subunits [53]. It is not clear whether orthologs of α and γ subunits exist in other mammals, as these proteins are members of the glandular kallikrein family of proteases [54], which has apparently evolved independently in mice and humans by duplication of an ancestral gene [55]. Even in mice, the existence of the 7S-NGF complex has not been clearly established in various tissues serving as physiologically important sources of NGF. Therefore, 7S-NGF appears to represent a specific adaptation of the mouse salivary gland that has little relevance elsewhere. Henceforth in this chapter the term “NGF” will be employed to refer to the protein originally known as “ β -NGF”.

X-ray crystallographic methods have been employed to determine the three-dimensional structure of NGF, NT-3 and NT-4 homodimers [56–58], and BDNF/NT-3 and BDNF/NT-4 heterodimers [58–60]. As might be expected from their substantial sequence similarity, the four neurotrophins fold similarly—three antiparallel pairs of β strands form a flat surface, which represents the interface of interaction of the two polypeptide chains. Six cysteinyl residues form three intrachain disulfide bonds, arranged in a characteristic cysteine-knot structure, which resembles structures found in several other growth factors, including PDGF-BB and transforming growth factor β (TGF- β)1 [61] and more closely resembles a cysteine-knot structure in the *Drosophila* toll-like receptor ligand Spätzle [62].

Mutagenesis studies have mapped amino acid residues in neurotrophins that are critical for binding to p75^{NTR} and Trk receptors. In each neurotrophin a cluster of positively charged lysyl residues forms an important binding interface for interactions with p75^{NTR} [63] and also contributes to the binding of NT-3 to its nonpreferred Trk receptors TrkA and TrkB [64]. Association of NGF with TrkA and BDNF with TrkB is mediated by amino acid residues grouped on one side of the neurotrophin dimer, forming a continuous surface for interaction with the Trk receptors [65]. The importance of these interaction domains was confirmed by subsequent determination of the three-dimensional structure of neurotrophin/receptor complexes by X-ray crystallographic methods [66–68]. These studies will be discussed in greater detail below.

9.4 STRUCTURE OF NEUROTROPHIN RECEPTORS

9.4.1 Structure of p75^{NTR}

p75^{NTR} was the founding example of the NGF/tumor necrosis factor (TNF) receptor gene superfamily, a family with some two dozen members in humans. These receptors are all single-pass membrane proteins, with the aminoterminal located extracellularly. The family is defined by a characteristic cysteine-rich repeat motif, first recognized in p75^{NTR} [13, 14], which represents the ligand binding site of the extracellular domain [69–72]. p75^{NTR} possesses four cysteine-rich repeat motifs; the number of these repeats in other members of the superfamily varies from one to four. Curiously, while members of this receptor superfamily generally signal as homotrimers and bind TNF-related

trimeric ligands [71], p75^{NTR} forms dimers rather than trimers [68, 73] and binds neurotrophins, which are dimeric and are unrelated in structure to TNF. The structure of the cysteine-rich repeat domain of a p75^{NTR} monomer complexed to NGF has been determined by crystallographic methods [68, 74]. These studies reveal that the four cysteine-rich repeats are extended more or less linearly. This elongated structure interacts along its length with the homodimeric interface of NGF. The two studies yield conflicting conclusions concerning whether dimeric NGF can mediate dimerization of p75^{NTR}. The serine/threonine-rich neck region that links the cysteine-rich repeat domain to the membrane-spanning domain of the receptor is not represented in these crystal structures. This neck region, which is poorly conserved across species and is therefore probably relatively unstructured, is modified by O glycosylation [75] while N glycosylation modifies the cysteine-rich repeat domain [13, 14, 73].

The most notable feature in the cytoplasmic domain of p75^{NTR} is a so-called death domain [76, 77], an amphipathic helical motif that typically mediates association with other death domain-containing proteins. Although such domains commonly participate in apoptotic cell death signaling pathways, the death domain of p75^{NTR} apparently does not function in this manner (see below). p75^{NTR} also possesses a C-terminal (Ser-x-Val) motif, which represents a postsynaptic density 95, disk large, zona occludens-1 (PDZ) domain binding site [78].

Recently structural homologs of p75^{NTR} have been identified. Zebrafish and *Xenopus* possess a homolog known as neurotrophin receptor homolog-1 (NRH1) [79] or fullback [80], which shows substantial sequence similarity to p75^{NTR} in cysteine-rich repeat, transmembrane, and death domains and, like p75^{NTR}, possess a C-terminal PDZ domain binding motif. The function of NRH1 is poorly understood and no ligand has been identified. However, NRH1 is apparently essential for the convergent extension process during gastrulation in *Xenopus* [81]. The mammalian p75^{NTR} homolog, known as PLAIDD [82], NRH2 [79], or NRADD [83], is the ortholog of NRH1 (Mark Bothwell and Harald Frankowski, manuscript in preparation), although it differs from NRH1 in one important respect. NRH2 lacks the cysteine-rich repeat domain but shares extensive similarity with NRH1 in transmembrane domain and cytoplasmic domains. NRH2, like NRH1 and p75^{NTR}, possesses a C-terminal PDZ domain binding motif.

9.4.2 Structure of Trk Receptors

Like other receptor tyrosine kinases, Trk receptors span the membrane once, with the amino terminus located extracellularly. The extracellular domain of Trk receptors is comprised by an array of three tandem leucine-rich motifs flanked by two cysteine-rich clusters and on the C-terminal side of this regions are two repeats of an immunoglobulin-like domain [84]. Mutagenic studies have led to discrepant conclusions concerning the location of the neurotrophin binding site. Most studies indicate that neurotrophins bind to the second immunoglobulin-like domain [85–90] while several suggest that neurotrophins bind to the leucine-rich repeat region [91–93]. The interaction of neurotrophins with the second immunoglobulin-like domain has been confirmed by X-ray crystallographic studies of a complex of NGF with the second immunoglobulin-like domain of TrkA [66, 67]. As the leucine-rich repeats were not present in the crystals employed, a role of leucine-rich repeats in neurotrophin binding cannot be entirely excluded by these studies. However, a subsequent

crystallographic study employing a complex of NGF with a TrkA extracellular domain fragment containing the leucine-rich repeat region as well as both immunoglobulin-like domains showed no contacts between the leucine-rich repeat region and NGF (Christopher Garcia, Stanford University, personal communication).

The cytoplasmic domain is highly conserved among the three Trk receptors and 10 tyrosyl residues are conserved, many of which are phosphorylated by the activated tyrosine kinase domain. The nature of the participation of these sites will be discussed in the following section. Discussion of the structure and function of Trk receptors is complicated by the existence of multiple splice variant forms of these receptors. The most striking of these include variant forms of TrkB [94, 95] and TrkC [96, 97] that possess shortened cytoplasmic domains lacking a tyrosine kinase domain. The functional significance of these alterations will be discussed in a later section.

9.5 SIGNALING BY TRK RECEPTORS

9.5.1 Determinants of Neurotrophin Preference

While TrkC functions exclusively as a receptor for NT-3 [30], TrkA and TrkB each can be activated by several neurotrophins. TrkA is activated by both NT-3 and NGF [98], while TrkB is activated by NT-3, BDNF, and NT-4 [27–29]. The preference of TrkA and TrkB for particular neurotrophins is regulated by two different mechanisms. First, alternative splicing generates several forms of the extracellular domains of TrkA and TrkB with differing neurotrophin preference. A variant inserting a short sequence in the extracellular domain of TrkA promotes activation by NT-3, without affecting activation by NGF [99]. A similar insert in TrkB promotes activation by NT-3 and NT-4, while TrkB, lacking this insert, is activated only by BDNF [100, 101]. Second, association of p75^{NTR} with TrkA suppresses activation of TrkA by NT-3 [99, 102–104] while promoting activation by NGF [105]. Similarly, association of p75^{NTR} with TrkB permits activation by BDNF but suppresses activation by NT-3 and NT-4 [106].

9.5.2 Signal Transduction Pathways

Like other receptor tyrosine kinases, Trk receptors are activated by neurotrophin-induced dimerization [66, 107], which leads to autophosphorylation of tyrosyl residues in the cytoplasmic domain. These sites of tyrosine phosphorylation can be placed into two functional categories. One category of tyrosyl residues, when phosphorylated, serve as docking sites for various signaling proteins, so that preferential phosphorylation of particular sites may determine which signaling pathway predominates. A second category of tyrosyl residues, located in the so-called activation loop, must be phosphorylated to permit phosphorylation of tyrosyl residues in the first category [108].

The signaling mechanism of TrkA has been investigated more thoroughly than the signaling mechanisms of TrkB or TrkC, but considering the near-perfect conservation in the number and placement of tyrosyl residues among the three Trk receptors, it is likely that observations made for TrkA can generally be extrapolated to TrkB and TrkC, and where data are available for TrkB and TrkC, they generally support this conclusion. In TrkA, the most important sites of tyrosine phosphorylation, outside of the activation loop, are tyrosine (Y) 485, which binds phospholipase C γ -1

(PLC γ -1) [109], and Y490, for which two signaling adapter proteins, Frs2 and Shc, compete for binding [110, 111].

PLC γ -1 is phosphorylated and activated following binding to phosphorylated Y485 of TrkA. Activated PLC γ -1 hydrolyzes the plasma membrane lipid phosphatidyl inositol(4, 5)P₂, generating inositol tris-phosphate (IP₃) and diacylglycerol (DAG) (reviewed in [112]). IP₃ promotes release of Ca²⁺ from internal stores, impacting a variety of cell functions, while DAG activates several isoforms of protein kinase C. BDNF binding to TrkB activates PLC γ -1 by a mechanism that is equivalent to that of NGF acting on TrkA [113, 114].

Association of Shc with phosphorylated Y490 of activated TrkA leads to phosphorylation of Shc and association of Shc with Grb-2 and son-of-sevenless (SOS), a guanosine triphosphate (GTP) exchange factor that activates Ras [115]. Activating Ras induces Raf-mediated phosphorylation and activation of MEK1/MEK2, which phosphorylate and activate the mitogen-activated protein (MAP) kinases ERK1 and ERK2. Association of Frs2 with phosphorylated Y490 provides an alternative mechanism for activation of Ras. In this pathway, Trk-mediated phosphorylation of Frs2 causes Frs2 to bind Grb-2, which activates Ras through SOS, as above. In addition, however, Frs2 mediates a Ras-independent pathway, leading to activation of ERK1/ERK2. Tyrosine phosphorylated Frs2 binds Crk [110]. Activated Crk activates the GTP exchange factor CRK SH3-binding guanine nucleotide-releasing protein (C3G), which activates the G protein Rap1. Rap1 activates B-Raf, which initiates the MEK1/MEK2 ERK1/ERK2 cascade. While the Shc-dependent pathway produces transient activation of ERK1/ERK2, the Frs2-dependent pathway leads to more prolonged activation of ERK1/ERK2 [116, 117]. The kinetics of ERK1/ERK2 are functionally important, as transient ERK1/ERK2 activation promotes proliferation of neuronal precursor cells, while prolonged activation, which allows nuclear accumulation of ERK1/ERK2, promotes neuronal differentiation [117, 118]. The differing contribution of Shc and Frs2 to prolonged activation of ERK1/ERK2 is not the only important functional distinction between these two signaling adapter proteins. Phosphorylated Frs2 also recruits to TrkA the protein phosphatase SH2 domain containing phosphatase 2 (SHP2) [110], which is essential for NGF-dependent activation of ERK1/ERK2 [119].

Another key signaling pathway of Trk receptors employs PI3 kinase, which converts phosphatidyl inositol(4,5)P₂ (PIP₂) to phosphatidyl inositol(3,4,5)P₃ (PIP₃). PIP₃ activates phosphatidylinositol-dependent protein kinase (PDK-1), which phosphorylates and activates the protein kinase Akt, a key regulator of cell survival. Trk receptors employ three alternative pathways to activate PI3 kinase. In one pathway, PI3 kinase is activated by Ras following Grb-2-dependent Ras activation [120]. In a second pathway, Grb-2 recruits Gab1 or Gab2, which binds and activates PI3 kinase [121]. A third pathway, which is independent of Grb-2, involves Trk-mediated phosphorylation of insulin receptor substrates IRS-1 and IRS-2, which bind and activate PI3 kinase [122]. The IRS-1/2 association with TrkA is mediated, at least in part, by phosphorylated Y490. These signaling interactions are illustrated in Fig. 9.1.

9.5.3 Other Signaling Proteins That Associate with Trk Receptors

Activated TrkA, TrkB, and TrkC each binds atypical protein kinase C interacting protein p62. This interaction has been found to promote trafficking of Trk receptors

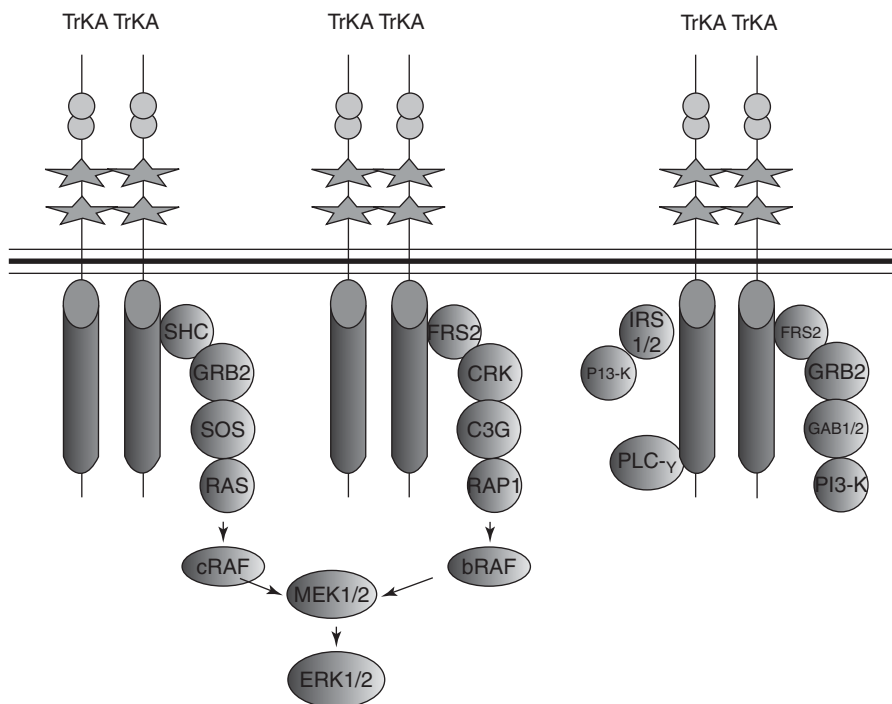


Figure 9.1 Trk receptors employ multiple signaling pathways. Dimerization of Trk receptors and resulting autophosphorylation of receptor tyrosyl residues recruit a variety of signaling proteins, as illustrated here for TrkA. Phosphotyrosine 490 alternatively binds Shc, Frs2, or IRS-1/IRS-2. Shc binds Grb2, while Frs2 binds either Grb2 or Crk. Grb2 binding causes activation of MAP kinases ERK1 and ERK2 by activating the GTP exchange factor SOS, which activates Ras, leading to activation of cRaf and thereby activation of MEK1/MEK2. Crk binding causes activation of ERK1 and ERK2 by recruiting the GTP exchange factor C3G, which activates Rap1, leading to activation of bRaf and thereby activation of MEK1/MEK2. Grb2 also binds and activates Gab1/Gab2, which activates PI3 kinase. IRS-1/IRS-2 also binds and activates PI3kinase.

to an endosomal compartment that facilitates activation of the MAP kinase ERK5 [123]. All three Trk receptors, when activated, also bind and tyrosine phosphorylate ankyrin-repeat membrane-spanning protein (ARMS). This association recruits binding of a complex of CrkL-C3G, which promotes Rap1-mediated sustained activation of ERK1/ERK2 [124].

The structurally related signaling adapter proteins SH2-B and adapter protein with PH and SH2 domains (APS) associate with phosphotyrosine residues within the tyrosine kinase domain of activated TrkA, TrkB, and TrkC and promote signaling by these receptors [125]. SH2-B apparently acts, at least in part, by promoting and prolonging the NGF-induced autophosphorylation of TrkA [126]. SH2-B may potentially also promote signaling in other ways since SH2-B binds IRS-1 and IRS-2 and promotes IRS-1/IRS-2-dependent PI3 kinase activation during leptin signaling [127]. In PC12 cells, overexpression of SH2-B promotes NGF-induced neurite outgrowth, while expressing an SH2-B mutant that cannot bind to TrkA inhibits NGF-induced neurite outgrowth, and in sympathetic neurons, disruption of

SH2-B function causes axon degeneration and death [126]. While these results seem to suggest that SH2-B is an essential mediator of NGF action, that conclusion may not be warranted as neither SH2-B null mice nor APS null mice have been reported to have any deficit in neuronal development [128, 129].

TrkA receptors directly bind to one important target of functional regulation, vanilloid receptor 1 (VR1), a heat-sensitive calcium channel also known as the capsaicin receptor. NGF is known to potently elicit sensations of pain by sensitizing the skin to noxious stimuli. An important element of this function is sensitization of VR1 channels by TrkA-mediated activation of PLC γ . VR1 is inhibited by PIP2, and depletion of PIP2 by PLC γ relieves this inhibition [130].

9.5.4 Influence of Trk Splice Variants on Signaling

As previously discussed, variations in the extracellular domains of Trk receptors produced by alternative splicing of transcripts influence the selectivity of receptors for neurotrophin binding. Other examples of alternative splicing influence Trk signaling more directly. For TrkA, a variant spliced mRNA has been described that does not encode exons 6, 7, or 9 and, consequently, lacks the first immunoglobulin (Ig)-like domain as well as sites for N glycosylation. As a consequence of these differences, the encoded protein, TrkAIII, is constitutively activated for signaling through PI3 kinase and PLC γ but does not signal through the ERK1/ERK2 pathway and does not bind NGF. TrkAIII is preferentially expressed in neural progenitor cells and in neuroblastomas, where its preferential signaling through the PI3 kinase pathway is suggested to promote an oncogenic effect [131]. It is interesting to note that pharmacological inhibition of N glycosylation of TrkA causes retention of TrkA in the endoplasmic reticulum and causes constitutive activation and signaling through the PLC γ pathway but not the ERK1/ERK2 pathway [132]. This suggests that TrkAIII may also be retained within the endoplasmic reticulum, explaining its failure to bind NGF.

Multiple splice variant forms of TrkB and TrkC affecting the structure of the intracellular domain have been described. TrkB produces three such variants, TrkB-T1, TrkB-T2 [133], and TrkB-T-Shc. TrkB-T1 and TrkB-T2 possess only short cytoplasmic domains that lack obvious signaling-related structural motifs. TrkB-T-Shc, on the other hand, retains a Shc binding site, but the functional relevance of this binding potential for Shc-mediated signaling has not been established. TrkB-T1 and TrkB-T2 are capable of acting as inhibitors of signaling through the full-length form (i.e., the tyrosine kinase domain-containing form) of TrkB, either by sequestering neurotrophins or by forming nonfunctional heterodimers [134]. On the other hand, TrkB-T1 and TrkB-T2 are capable of positive TrkB-independent signaling [135, 136], at least in part through association with p75^{NTR} [137]. These findings that some functions of truncated TrkB are independent of full-length TrkB is consistent with the observation that there are substantial differences in the spatiotemporal pattern of expression of these proteins during development. Whereas TrkB is expressed most highly in neurons, TrkB-T1/T2 is most abundant in glia and other nonneuronal cells [138–141].

Analogous to TrkB-T1 and TrkB-T2, five TrkC splice variants lacking a tyrosine kinase domain have been identified [96, 97, 142–144]. TrkC splice forms possessing and lacking a functional tyrosine kinase domain are extensively coexpressed in the brain [141, 145]. Like truncated forms of TrkB, truncated forms of TrkC have been

suggested to function as inhibitors of kinase domain-containing forms [146]. Also like truncated TrkB forms, however, TrkC variants lacking a tyrosine kinase domain have the capacity to signal in their own right and, like the TrkB system, signaling by a truncated TrkC form may be mediated by interactions with $p75^{\text{NTR}}$ [147].

In addition to TrkC variants that entirely lack a functional tyrosine kinase domain, two TrkC variants, TrkC K2 and TrkC K3, possess short inserted sequences within the tyrosine kinase domain that alter the signaling capacity of the receptors [97, 142]. These variants have diminished tyrosine kinase activity [97, 142], fail to bind Shc or PLC γ , and exhibit attenuated activation of ERK1/ERK2 [148].

9.6 $p75^{\text{NTR}}$ SIGNALING

The similarity of Trk signaling pathways to pathways employed by other receptor tyrosine kinases has greatly aided elucidation of Trk signaling. In contrast, progress in characterizing the signaling pathways employed by $p75^{\text{NTR}}$ has been much slower, because, although the similarity of $p75^{\text{NTR}}$ to other members of the NGF/TNF receptor superfamily has provided a few clues [149], most elements of $p75^{\text{NTR}}$ signaling differ markedly from other members of the superfamily. Furthermore, the signaling functions of $p75^{\text{NTR}}$ are remarkably complex. For example, regulation of apoptotic cell death is a major function of $p75^{\text{NTR}}$. However, in some systems, $p75^{\text{NTR}}$ constitutively promotes cell death, unless this proapoptotic signaling is inhibited by binding neurotrophin [150, 151]. In other systems, proapoptotic signaling by $p75^{\text{NTR}}$ is initiated by binding a neurotrophin or proneurotrophin [31, 152], while in still other systems, neurotrophin-induced antiapoptotic signaling by $p75^{\text{NTR}}$ is required to maintain cell survival [153, 154].

The Jun N-terminal kinase (JNK) protein family (JNK1, JNK2, JNK3) is an important mediator of proapoptotic signaling by $p75^{\text{NTR}}$ [155, 156]. $p75^{\text{NTR}}$ -induced JNK activation promotes cell death by phosphorylation of the protein Bad [155] and by induction of expression of p53 [157]. Association with $p75^{\text{NTR}}$ of the cytoplasmic protein NGF receptor interacting factor (NRIF) mediates activation of JNK [158] by a pathway that has not been fully characterized. $p75^{\text{NTR}}$ -mediated activation of sphingomyelinase activity [159], producing ceramide, has been implicated as a mechanism of JNK activation [156]. $p75^{\text{NTR}}$ also binds other proapoptotic signaling proteins, including NGF receptor binding MAGE protein (NRAGE) [160] and other members of the melanoma antigen gene (MAGE) gene family [161, 162] and NADE, a member of the Bex gene family [163]. Although NGF receptor associated effector (NADE), MAGE proteins, and NRIF have little in common structurally, they do share the functional property of shuttling from the cytoplasm to the nucleus. Consequently, the recent discovery that sequential cleavage of $p75^{\text{NTR}}$ by a disintegrin, and metalloproteinase 17 (ADAM17) and γ -secretase proteases releases the cytoplasmic domain of $p75^{\text{NTR}}$ into the cytoplasm and nucleus [79, 164, 165] potentially provides a mechanism for regulating the trafficking and function of these proapoptotic proteins.

Antiapoptotic signaling by $p75^{\text{NTR}}$ is mediated by several signaling pathways, including activation of the PI3 kinase/Akt pathway [166] and activation of NF-kappaB [154, 167]. The mechanism responsible for $p75^{\text{NTR}}$ -dependent activation of PI3 kinase is unclear. It has been suggested that $p75^{\text{NTR}}$ -mediated ceramide synthesis is required for activation of PI3 kinase [168], a perplexing notion since ceramide also has been put

forward as the mediator of proapoptotic signaling by p75^{NTR}. Activation of NF-kappaB by p75^{NTR} is mediated by association of p75^{NTR} with several signaling adapter proteins, including TRAF6 and other members of the TRAF gene family [169–171], and association with receptor interacting protein 2 (RIP2) [153]. TRAF6-dependent activation of NF-kappaB by p75^{NTR} involves association with a similar constellation of proteins as employed by the interleukin IL-1 receptor, including the signaling adapter protein Myd88 and the protein kinase IL-1 receptor associated kinase (IRAK) [171].

Another important signaling pathway of p75^{NTR} regulates activity of RhoA. p75^{NTR} constitutively activates RhoA, while NGF binding to p75^{NTR} suppresses this activation [172]. The ability of NGF binding to p75^{NTR} to promote signaling by one set of pathways (leading to NF-kappaB activation, for example) while suppressing signaling by another set of pathways (including RhoA activation) raises the fascinating proposition that p75^{NTR} is constantly engaged in signaling and changes the mode of signaling, rather than the extent of signaling, when neurotrophins bind. In the presence of the membrane protein Lingo-1 [173], p75^{NTR} associates with a GPI-linked protein known as Nogo receptor (NgR). The p75^{NTR}/Lingo-1/NgR complex activates RhoA and stimulates an increase in intracellular [Ca²⁺] in response to binding to NgR of several different membrane proteins of central nervous system myelin, including Nogo-A, MAG, and OMgp [174, 175]. Thus, p75^{NTR} mediates signaling by myelin proteins as well as by neurotrophins, and p75^{NTR} function is implicated in the inhibition of axonal regeneration in injured spinal cord by myelin proteins at the injury site. The inhibition of RhoA by one set of p75^{NTR} ligands (neurotrophins) and the activation of RhoA by another set of p75^{NTR} ligands (NgR associated with Nogo-A, MAG, or OMgp) underscores the curious yin/yang nature of p75^{NTR} signaling.

Pro-NGF and pro-BDNF have enhanced affinity for p75^{NTR} and reduced affinity for their cognate Trk receptors, relative to the corresponding mature neurotrophins [152]. As a significant fraction of secreted neurotrophin may be in the pro form in some systems, the extent of proteolytic processing that occurs following secretion may determine the relative extent of activation of p75^{NTR} versus Trk receptors [176]. The preferential association of proneurotrophins with p75^{NTR} is mediated by association of proneurotrophins with the protein Sortilin. Thus, the presence or absence of Sortilin expression also determines whether proneurotrophins preferentially activate p75^{NTR} versus Trk [177]. Note, however, that some studies do not confirm the preferential activation of p75^{NTR} by pro-NGF [178]. The distinct signaling functions of p75^{NTR} within complexes with Trk, Sortilin, or NgR/Lingo-1 suggest a modular organization of p75^{NTR} signaling functions, as illustrated in Fig. 9.2.

9.7 FUNCTIONAL INTERACTIONS OF p75^{NTR} AND TRK RECEPTORS

The functions of p75^{NTR} and Trk receptors are linked at multiple levels. As already mentioned, the association of p75^{NTR} with Trk receptors changes the neurotrophin specificity for Trk activation, for example, by increasing the affinity of NGF binding to TrkA. Although it might seem obvious that this effect is mediated by p75^{NTR} and TrkA jointly binding the same neurotrophin as many studies have speculated, a p75^{NTR} deletion mutant lacking the NGF binding site also increases the affinity of NGF binding to TrkA, indicating that p75^{NTR} influences TrkA ligand affinity by an

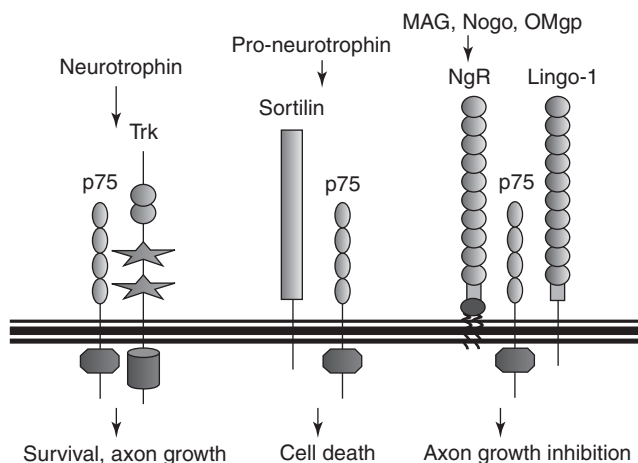


Figure 9.2 $p75^{\text{NTR}}$ signaling modules. Complexes of $p75^{\text{NTR}}$ with Trk receptors are implicated in neurotrophin-mediated neurotrophic signaling pathways. Complexes of $p75^{\text{NTR}}$ with Sortilin are preferentially activated by the proneurotrophins and signal by pathways promoting cell death. Complexes of $p75^{\text{NTR}}$ with Lingo-1 and NgR mediate inhibition of axon regeneration by myelin proteins such as Nogo, MAG, and OMgp.

allosteric effect on TrkA conformation [179]. The influence of $p75^{\text{NTR}}$ on affinity of TrkA for NGF binding suggests that $p75^{\text{NTR}}$ should enhance TrkA signaling, at least subsaturating concentrations of NGF, and that has been reported [105]. On the other hand, at receptor-saturating concentrations of neurotrophins, the presence of $p75^{\text{NTR}}$ actually suppresses TrkA signaling [180].

The $p75^{\text{NTR}}$ /Trk interaction also influences $p75^{\text{NTR}}$ function, either directly, by regulating $p75^{\text{NTR}}$ cleavage [79], or indirectly, by interactions of the signaling pathways of $p75^{\text{NTR}}$ and Trk receptors. Trk receptor signaling blocks $p75^{\text{NTR}}$ -mediated production of ceramide [181] and blocks $p75^{\text{NTR}}$ -mediated activation of JNK [182]. Thus, in cells coexpressing $p75^{\text{NTR}}$ with a Trk receptor, neurotrophin-induced apoptosis is typically observed only for neurotrophins that selectively activate only the $p75^{\text{NTR}}$ receptor. For example, in sympathetic neurons that express $p75^{\text{NTR}}$ and TrkA, BDNF induces apoptosis, whereas NGF does not.

The association of $p75^{\text{NTR}}$ with Trk receptors and interactions of the signaling pathways of these receptors may be mediated in part by signaling adapter proteins that bind jointly to $p75^{\text{NTR}}$ and Trk. Examples of signaling adapter proteins reported to associate with both $p75^{\text{NTR}}$ and Trk receptors include ARMS [183], Shc [184], and the MAGE protein Necdin [185]. A more complex manifestation of this principle involves the assembly of a multiprotein signaling platform recruiting atypical protein kinase C by the association with TrkA of the atypical protein kinase C binding protein p62 and association with $p75^{\text{NTR}}$ of the p62 binding protein Traf6 [186].

9.8 RETROGRADE AXONAL SIGNALING

In the common scenario envisioned in the “neurotrophic hypothesis,” neurotrophins released by an innervated target regulate survival of the innervating neurons by

binding to receptors on the terminals of axons from those neurons. As neuronal survival is typically dependent on apoptotic signals functioning in the neuronal cell bodies, this scenario demands that neurotrophin-dependent signals be transmitted from the axon terminus to the cell body, a distance that may exceed 1 m for some neurons in large mammals. A hint of a possible mechanism was discovered several decades ago by the finding that radiolabeled NGF could be transported from the axon terminus to the cell body [187], but the full significance of this finding did not become clear until recently. Extensive evidence now supports a signaling mechanism wherein neurotrophin/Trk receptors at the axon terminus undergo endocytosis, leading to the formation of specialized vesicles called signaling endosomes [188]. These endosomes, bearing an intact and active neurotrophin/Trk complex, are driven retrogradely along microtubules by dynein motor proteins [189], delivering active Trk receptors, with associated signaling proteins, to the vicinity of the nucleus in the cell body, where transcription factors such as cyclic adenosine monophosphate (cAMP)–responsive element binding protein (CREB) are activated [190–193].

Processes regulating the formation and trafficking of signaling endosomes are still poorly understood. The hidden complexity of this system is illustrated by the observation that neurotrophins may influence activation of cell surface receptors and formation of signaling endosomes differentially. For example, in both neurons and transfected nonneural cell lines, under circumstances where NT-3 and NGF activate cell surface TrkA receptors similarly, NGF induces endocytic internalization of TrkA, whereas NT-3 does not [194]. Although the presence of p75^{NTR} influences the relative efficacy of NGF and NT-3 for activating cell surface receptors, as described previously, it is not responsible for the differential internalization of TrkA with NT-3 and NGF. p75^{NTR} itself does contribute to retrograde transport of neurotrophins [195] and produces a signaling endosome [196] that is distinct from the Trk signaling endosome. However, the contribution of p75^{NTR} to retrograde axonal signaling is still unclear.

9.9 SUMMARY

The enormous functional complexity of the neurotrophin signaling system, which already is daunting in simple nonneuronal cell types, is even more impressive in neurons, where receptors potentially can be differentially targeted to different subcompartments, including dendritic synaptic sites and axon termini, and signal differently from those sites. Although the last several decades have seen huge advances in our understanding of neurotrophin function, it is clear that much still remains to be learned. One area where our ignorance is still profound concerns the mechanisms that achieve spatiotemporal specificity for neurotrophin secretion, for proper cell surface positioning of receptors, and for regulation of signaling behavior of endocytic vesicles. Another area which will no doubt see great advances in the future will be the replacement of the present concept of receptors signaling as lonely entities with a more systems-oriented understanding of the extensive functional interactions among membrane proteins and their signaling effector proteins.

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10

TOURETTE'S SYNDROME AND PHARMACOTHERAPY

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10.1	Introduction	263
10.2	Phenomenology	264
10.3	Clinical Course	266
10.4	Epidemiology	266
10.5	Comorbid Disorders	266
10.6	Genetics	266
10.7	Environment	267
10.8	Pathophysiology	269
10.8.1	Neuroanatomical Abnormalities	269
10.8.2	Neurochemical Abnormalities	270
10.8.3	Second-Messenger Systems	273
10.9	Treatment	273
10.9.1	Introduction	273
10.9.2	Neuroleptics	273
10.9.3	Other Antidopaminergic Agents	276
10.9.4	The α_2 Agonists	276
10.9.5	Other Tic Suppressants	277
10.9.6	Nonpharmacological Treatments	277
10.10	Future Directions	278
	References	278

10.1 INTRODUCTION

Tics are sudden, rapid, recurrent, nonrhythmic, stereotyped movements, gestures, or vocalizations, often mimicking some type of normal behavior. They are classified in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV-TR [1]) according to type—motor or phonic—and duration, being either transient or chronic (Table 10.1). Transient tics of childhood onset are characterized by a time-limited expression of one

TABLE 10.1 DSM-IV-TR Criteria for Tic Disorders

Tourette’s disorder
A. Both multiple motor and one or more vocal tics have been present at some time during the illness, although not necessarily concurrently.
B. The tics occur many times a day (usually in bouts) nearly every day or intermittently throughout a period of more than 1 year, and during this period there was never a tic-free period of more than three consecutive months.
C. The onset is before 18 years.
D. The disturbance is not due to the direct physiological effects of a substance (e.g., stimulants) or a general medical condition (e.g., Huntington’s disease or postviral encephalitis).
Chronic motor or vocal tic disorder
A. Single or multiple motor or vocal tics, but not both, have been present at some time during the illness.
B. The tics occur many times a day (usually in bouts) nearly every day or intermittently throughout a period of more than 1 year, and during this period there was never a tic-free period of more than three consecutive months.
C. The onset is before 18 years.
D. The disturbance is not due to the direct physiological effects of a substance (e.g., stimulants) or a general medical condition (e.g., Huntington’s disease or postviral encephalitis).
E. Criteria have never been met for TS.
Transient tic disorder
A. Single or multiple motor and/or vocal tics.
B. The tics occur many times a day, nearly every day for at least 4 weeks, but for no longer than 12 consecutive months.
C. The onset is before 18 years.
D. The disturbance is not due to the direct physiological effects of a substance (e.g., stimulants) or a general medical condition (e.g., Huntington’s disease or postviral encephalitis).
E. Criteria have never been met for TS or chronic motor or vocal tic disorder.
Tic disorder not otherwise specified: This category is for disorders characterized by tics that do not meet criteria for a specific tic disorder. Examples include tics lasting less than 4 weeks or tics with an onset after age 18 years.

Source: *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed., Text Revision, American Psychiatric Association, Washington, DC, 2000.

or more simple motor or phonic tics. Chronic tics are either motor or phonic tics, which may present as more severe than transient tics and last longer than one year. Tourette’s syndrome (TS) is characterized by both multiple motor and one or more vocal tics, which may appear simultaneously or at different periods during the illness and wax and wane in severity [2].

10.2 PHENOMENOLOGY

Although the age of onset for TS has been reported to be as early as 1 year old [3], motor tics usually begin between the ages of 3 and 8 years [4], with transient periods of intensive eye blinking or some other facial tics. Extreme cases, mostly seen in adulthood, may involve severe bouts of self-injurious tics such as biting or hitting as

well as complicated gestures. Phonic tics can begin as early as 3 years of age but typically follow the onset of motor tics by several years [4, 5]. They may consist of repetitive bouts of sniffing, grunting, or throat clearing or, more seriously, coprolalic utterances, shouting obscenities, or racial slurs. The median age of onset for tics is estimated to be 7 years [4] and onset after the age of 12 is highly unusual. However, cases of adult onset have been described [6].

Tics arise in bouts over the course of a day, changing in severity over weeks and months [7]. These episodes are characterized by stable intra-tic intervals (the time between successive tics) of short duration, typically 0.5–1.0 s. However, there is also a self-similarity of those patterns visible over time [8]. Over only minutes to hours, bouts of tics happen in groups, while over the course of weeks to months, many episodes of tics arise (Fig. 10.1). This periodic higher order combination of tic bouts could be the basis of the waxing and waning course of TS. Understanding these tic patterns is of importance for physicians treating TS, in particular with regards to their decision making about when to initiate antitic medication trials, change dosage, or be patient and provide close monitoring of symptoms instead.

Sensory phenomena, including premonitory urges, may precede tics, incessantly prompting tics with subsequent posttic feelings of momentary relief [9–11]. They are described as feelings of pressure, or an itch, impelling the person to perform a tic in order to relief the sensation. These urges may be as debilitating as the tics themselves. Auditory or visual cues (e.g., a cough, particular word, or specific shape) may also prompt tics.

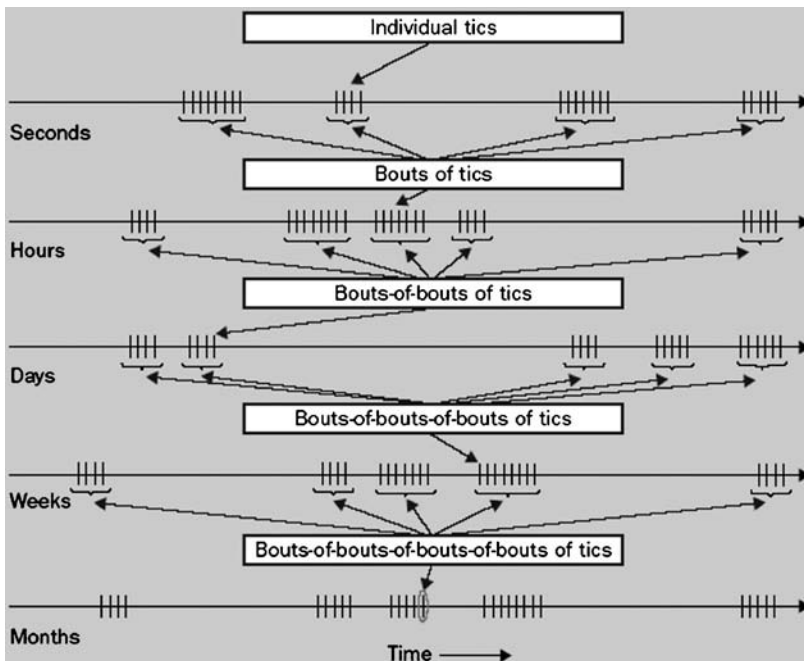


Figure 10.1 Fractal character of temporal occurrence of tics. Progressively longer time scales (seconds to months) are depicted. [With permission from J. F. Leckman, Tourette's syndrome, *Lancet* 2002;360(9345), 1577–1586.] (See color insert.)

10.3 CLINICAL COURSE

TS has typically a gradual onset, and its severity is often compounded by other, comorbid psychiatric disorders, in particular attention-deficit hyperactivity disorder (ADHD) [12] and obsessive compulsive disorder (OCD) [13, 14]. The waxing and waning course of TS may become less varied once patients reach adolescence [8], while overall tic severity, with periods of worst tics at around age 10, tends to diminish in early adulthood [4], disappearing in half of the patients by age 18 years. A study in 58 adult patients with TS [15] found that for most patients the worst functioning had occurred during adolescence (mode 13 years). Coprolalia, which is seen in approximately 30% of TS clinic patients and has an onset around age 15, persisted only in 4% of patients [16].

10.4 EPIDEMIOLOGY

Estimates of the prevalence of TS have varied over the years. Initially thought to be a rare disorder, depending on the characteristics of the study population, study design, and methods, estimates have ranged from 3 to 15 per 1000. Epidemiological studies in mainstream school populations indicate a prevalence ranging from 0.15 to 3.8% in the general population [17–22], with expression of tics consistently found to be higher among boys than girls and tics appearing in all social and ethnic groups [3, 14, 19, 23]. However, there are indications that interracial differences exist, with lower prevalence rates of TS in Asian versus Caucasian populations, as indicated by a small Japanese study [24].

10.5 COMORBID DISORDERS

Robertson [25] suggests subdividing TS into three groups. The first is “simple TS” with motor and phonic tics the predominant and almost only symptoms. Second is “full-blown TS” with copro and echo phenomena. Third is “TS plus” in which the patient may have ADHD, OCD, self-injurious behaviors, and a variety of other complex psychopathologies. In general-population samples the prevalence of ADHD is about 5% [26, 27], but this up to eightfold higher (17–38%) in subjects who also carry a diagnosis of TS or chronic tic disorder [17, 24, 28]. Several studies indicate that up to 60% of patients with TS also report OCD symptoms (OCSs) during the course of their illness [17, 29, 30], while up to 17% appear to meet DSM-IV-TR criteria for OCD [17, 29]. The OCSs generally become more prominent during adolescence and, occasionally, can become more burdensome than the tics. OCSs in TS are more often aggressive in nature than in OCD patients without tics [31], while tic-related forms of OCD are frequently associated with a need to repeat actions until they feel “just right” [10]. Comorbidity of depressive and anxiety disorders range from 9 to 29% for anxiety disorders [17, 32, 33] and from 10 to 23% for depressive disorders [17, 33].

10.6 GENETICS

Research in twins and families over the past two decades has shown that genetic factors are implicated in vertical transmission in families with vulnerability to TS.

However, the nature of the vulnerability genes predisposing individuals to TS is yet unknown. Characterization of the range of behaviors comprising the phenotype of TS may aid in identifying the specific vulnerability types. Endophenotypes are measurable aspects of a psychiatric disorder that can be used in linkage analysis as quantitative traits, modeled in animal studies, or both. Several studies using this technique have been published in the last decade, including descriptions of components for the OCD phenotype [34, 35], while recently a symptom component analysis contributed to finding the separate genetic loci for developmental dyslexia [36]. A factor analysis of TS revealed four factors accounting for 61% of the phenotypic symptom variance in TS: two factors predicting TS with comorbid ADHD, one comorbid OCD, and one motor and phonic tics, needed for a diagnosis of TS [37].

Segregation analysis is used to test specific genetic hypotheses regarding transmission of a disorder within families. Different genes are thought to be involved in TS. Review of six TS family studies showed evidence consistent with an autosomal dominant inheritance pattern in three families, while the most parsimonious solution for the other three was a model in which penetrance of heterozygous individuals was between homozygotes [38]. Another study found a significant multifactorial polygenic background in which the major locus accounted for more than half of the phenotypic variance [39].

Candidate gene studies have been assessed for TS, including dopamine receptors *DRD1*, *DRD2*, *DRD4*, and *DRD5*, noradrenergic genes *ADRA2a* and *ADRA2C* as well as serotonergic receptor gene *5HTT* [40, 41]. Although one study found evidence for linkage between TS and *DRD2* [40], further studies investigating this finding excluded the proposed linkage between the *A1* allele at the *DRD2* locus [42], while its significance for the severity of TS was also excluded [43]. Other studies excluded linkage between TS and *DRD1* [44], *DRD3* [45], *DRD4* [46, 47], *DRD5* [48], and the serotonin (5-hydroxytryptamine, or 5-HT) receptor-7 (*5HT7*) gene [49].

Genomic screens have also been used. These assume that a few so-called founder individuals contribute to the vulnerability gene, which is now distributed in a much larger population. The screens in affected sibpairs [50], multigenerational families [51], and a South African population [52, 53] implicated regions near the centromere of chromosome 2 and on 5p, 5q, 6p, 8q, 11q, 14q, 19p, 20q, and 21q. Thus far, only the involvement of 11q has been confirmed in a study involving a large French Canadian family [54].

10.7 ENVIRONMENT

Twin studies show that the concordance rate for TS in monozygotic twins is 56% [55], indicating that environmental or epigenetic factors also play a role in the pathogenesis of TS. Environmental factors associated with an increase for tic symptoms include pre- and perinatal events such as low birth weight [55], low Apgar scores [56], and maternal stress, including first-trimester nausea and vomiting [57]. Other environmental factors include anxiety-provoking events, emotional trauma, and social gatherings [58]. However, the latter study also found that environmental factors may be beneficial for the course of TS, including talking to friends and reading for pleasure. Further evidence for the role of stress in TS are findings that TS patients excrete more norepinephrine (NE) after lumbar puncture compared to

controls [59], while higher concentrations of NE and corticotropin-releasing factor in their cerebrospinal fluid (CSF) also have been found [60, 61].

While the previously discussed comorbid psychiatric disorders ADHD, OCD, depression, and learning disorders play a role in the pathogenesis of TS, medical disorders, in particular infections, may also play a role. A series of case studies in the literature reported sudden forms of tics and OCS [62, 63] linked in particular to poststreptococcal infections responding only to unconventional TS treatment methods such as plasma exchange, intravenously administered immunoglobulins [64], or antibiotics [62, 63]. Group A β -hemolytic streptococci are known to be a possible trigger of immune-related disease in genetically predisposed individuals, in particular Sydenham's chorea, a disorder thought to affect the basal ganglia and cortical and thalamic areas in the brain, the same anatomic areas affected by TS. In addition, some Sydenham's chorea patients present with tic, ADHD, and OCSs, suggesting a common cause between the two disorders, while, as in Sydenham's chorea, antineural antibodies have been found in some patients with TS [65–67]. All these factors led to an interest in autoimmune mechanisms as the contributing factor to TS and OCD and to the formulation of clinical criteria for PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections) by the National Institute of Mental Health [68]. The criteria include the presence of OCD and/or tics, sudden and prepubertal onset, and temporal association between streptococcal infections and symptom exacerbation. The criteria were formulated after studying a sample of 50 subjects with possible PANDAS. However, questions have been raised about the diagnostic criteria and their validity [69–71], in particular the lack of controlled studies to define the criteria [70]. Another concern raised is whether tic and related disorders should be called autoimmune disorders, even in the presence of a relationship with infections [69, 72]. The possibility of tic onset due to immune dysregulation was first reported in two studies describing positivity for D8/17 [73, 74], a specific monoclonal antibody to B lymphocytes found in 5% of normal controls but on average in 33.5% of individuals with rheumatic fever [75]. D8/17 positivity was almost diagnostic for a tic disorder or OCD with 85–100% of patients positive [73, 74]. Only 5–15% of controls were positive. The method by which positivity was assessed, immunofluorescence, was called into question, however [72], since more objective measures found group differences between patients with tics and OCD and healthy controls [76–78]. In addition, assessment through MOC32, an immunoglobulin M (IgM) monoclonal antibody directed against a neuroendocrine antigen of epithelial origin of small-cell lung cancer cells, indicated that there was no overexpression of D8/17 on B cells in tic disorder patients compared to controls. Rather, it detected increased expression of receptors for the constant part of IgM molecules (Fc- μ) on B cells, more likely indicative of generalized immune activity [77]. Further evidence against involvement of D8/17 is a Mexican study [79] which failed to provide support for the use of D8/17 as a marker of susceptibility to tics and OCD in a community sample of 240 children of which 108 were positive and 132 negative for D8/17. Finally, a study measuring antistreptococcal antibody (ASA) titers in 105 subjects with chronic tics, OCD, or ADHD and 37 healthy controls [80], all without known history of streptococcal exposure, showed no relationship between ASA titers and a diagnosis of tics or OCD. However, analyses of the basal ganglia volumes indicated significant differences between OCD and ADHD subjects versus tic subjects and healthy controls, with larger volumes of putamen and globus pallidus (GP) associated with higher ASA,

suggesting that the relationship between ASA and tics or OCD may have been confounded by ADHD.

Evidence of autoantibodies reacting with parts of the brain involved with tic disorders appears to be stronger evidence for an autoimmune hypothesis of tic disorders [69]. Two studies [65, 74] showed positive staining in 44–50% of subjects with TS and/or OCD versus 21–24% in healthy controls, while enzyme-linked immunosorbent assay techniques against immortalized neuronal cell line [81, 82] and human basal ganglia [67] confirmed increased levels of serum antineuronal antibodies in TS patients.

10.8 PATHOPHYSIOLOGY

Research in the pathophysiology of TS generally focuses on abnormalities in the neuroanatomical sites involved with TS (e.g., within the corticostriatothalamocortical pathway), in physiological abnormalities (e.g., disinhibited afferent thalamocortical signals), and in neurochemical abnormalities (i.e., at the cellular level) [83].

10.8.1 Neuroanatomical Abnormalities

Habits are assembled routines, linking sensory cues with motor action, and are thought to be essential for the understanding of TS [84–87]. Circuits involved with habit forming are the motor, sensorimotor, association, inhibitory, and limbic neural circuits that course through the basal ganglia [85–88]. These circuits form multiple cortical–subcortical loops, directing information from the cerebral cortex to the cortex and then back to specific regions of the cortex (Fig. 10.2). Abnormalities in these circuits (e.g., disinhibition of excitatory neurons in the thalamus) may result in hyperexcitability of cortical motor areas and the release of tics.

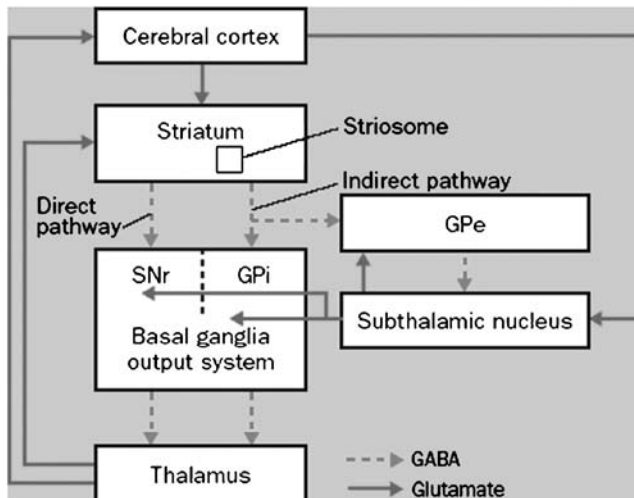


Figure 10.2 Schematic diagram of major connections of basal ganglia: GPe, globus pallidus, pars externa; Gpi, globus pallidus, pars interna; SNr, substantia nigra, pars reticulata. [With permission from J. F. Leckman, Tourette's syndrome, *Lancet* 2002;360(9345), 1577–1586.] (See color insert.)

Cortical neurons projecting to the striatum outnumber striatal neurons by about a factor 10 [89] and segregate in two neurochemically different compartments, striosomes and matrix (Fig. 10.3). The cortical inputs for these compartments differ; the striosomal medium receives convergent limbic and prelimbic input, while the matrix receives convergent input from ipsilateral motor and sensorimotor cortices and the contralateral primary motor cortex. Both are innervated by distinct subcortical afferents; however, striosomal axons project to the substantia nigra pars compacta (SNpc), while matrix axons project to the GP. Since the striatum has a significant role in the production of motor stereotypies, developmental changes in density in the neurotransmitter uptake sites and in compartmental receptor specificity in the striatum [90–92] may contribute to motor stereotypies in TS; however, they can also be caused by agonists to dopamine (a neurotransmitter present in abundance in the striatum) such as apomorphine [93]. Under normal circumstances the highest peak metabolic activity is in the matrix [87]. An imbalance in the matrix/striosomal activities can also cause tics and stereotypies [94, 95].

Although structural changes in the basal ganglia of TS patients have been reported [96], broadly distributed cortical systems may be more important determinants of tics and hyperkinetic behaviors [97–100].

10.8.2 Neurochemical Abnormalities

Therapeutic responses to most neurotransmitters in the corticostriatothalamocortical circuit, including dopamine (DA), 5-HT, choline, NE, glutamate, γ -aminobutyric acid (GABA), and opioids, as well as evidence provided by postmortem brain tissue studies, positron emission tomography (PET) studies, single-photon emission computed tomography (SPECT) studies, and CSF, blood, and urine studies, have led to hypotheses concerning neurotransmitter imbalances as primary biological factors contributing to TS [83].

Evidence for disturbance in DA neurotransmission stems from the fact that the majority of TS patients report significant symptom improvement when treated with neuroleptic medication selectively blocking D_2 receptors [101, 102] while withdrawal from neuroleptic treatment or exposure to medications that increase central dopaminergic activity such as central nervous system (CNS) stimulants often worsens tics [103–105]. Further support comes from CSF studies indicating altered levels of the DA metabolite homovanillic acid (HVA) in TS subjects compared to controls [104, 106, 107]. Based on these findings DA receptor hypersensitivity was hypothesized. However, a more recent study did not replicate the findings [61], while postmortem studies assessing DA1 and DA2 striatal tissue receptor binding differences between TS subjects and controls [108, 109] and PET and SPECT studies assessing DA hypersensitivity [91, 110–112] or hyperinnervation [92, 113–116] showed conflicting results. Finally, an overactive DA transporter system has been proposed [115, 117] which would create reduced levels of extracellular DA, higher concentrations of DA in the axon terminal, increased stimulus-dependent DA release, autoreceptor supersensitivity at the presynaptic site, and increased sensitivity to low-dose neuroleptics. Supporting this hypothesis is stimulant-induced tic exacerbation [103, 105, 118], which could be due to greater DA release from the axon terminal, as well as tic suppression with very low dose neuroleptics, possibly due to availability of a reduced amount of DA to be blocked.

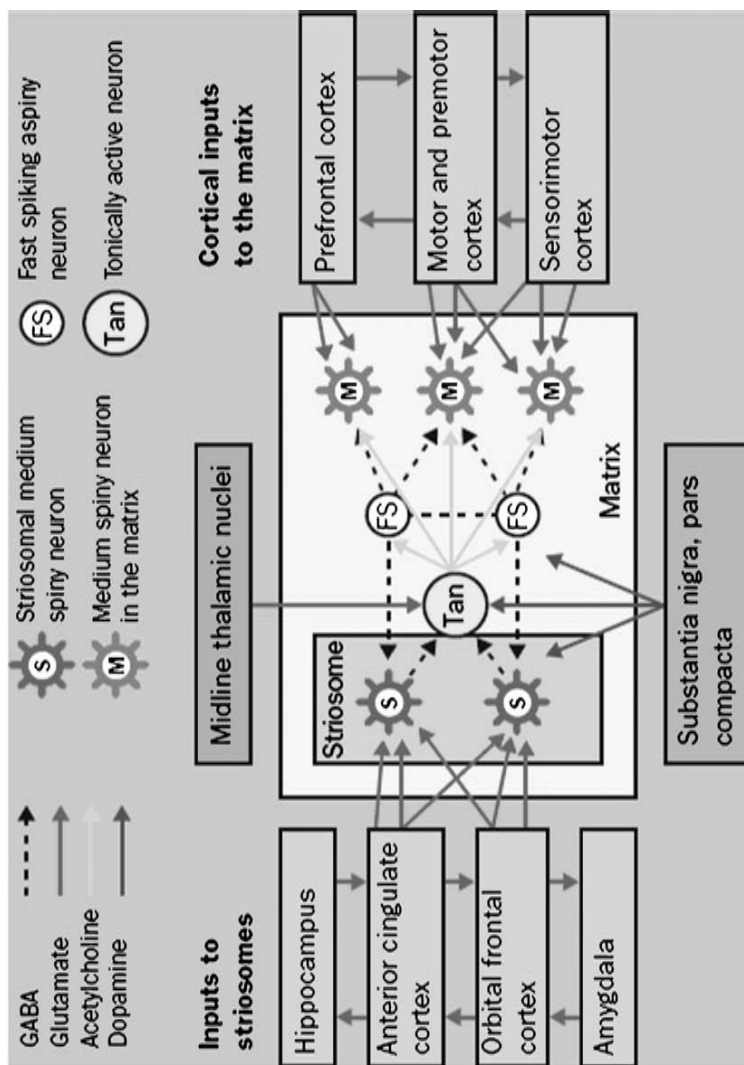


Figure 10.3 Schematic diagram of major inputs into medium spiny GABAergic projection neurons of striatum. [With permission from J. F. Leckman, Tourette's syndrome, *Lancet* 2002;360(9345), 1577–1586.] (See color insert.)

The involvement of 5-HT in TS has been studied since it plays an important role in the neurophysiology of OCD, a comorbid disorder in many TS patients. However, to date there is minimal supportive evidence suggesting a role for 5-HT in the pathophysiology of TS. Studies assessing the presence of 5-hydroxyindoleacetic acid (5-HIAA), the principal metabolite of 5-HT, have shown only minimal or no reduction in the levels of CSF 5-HIAA in subject with TS [61, 104, 106], but tryptophan, the 5-HT precursor, may be decreased in basal ganglia [119, 120], while a reduction in plasma tryptophan has been reported in unmedicated TS patients [121]. Furthermore, 5-HT transporter protein has been shown to be reduced in subjects with OCD and comorbid TS but not in TS alone. Hence, it may be that 5-HT has a modifying rather than causative role in TS.

Few studies have investigated the role of glutamate in TS. However, several recent studies indicate that glutamatergic hyperactivity may be a causative factor. Pentylenetetrazole administered to mice expressing a neuropotentiating transgene in a cortical limbic subset of D_1 + neurons, known to trigger glutamatergic excitation of orbitofrontal, sensorimotor, limbic, and efferent striatal circuits, caused an increase in seizure activity. Since seizures, OCD, and tics often coexist, the authors suggested a dopamine-regulated glutaminergic bases for all three states [122]. Further, possibly more convincing evidence for the role of glutamate in TS was shown in a study indicating increased tic behavior, tic complexity, tic bouts, and evidence for juvenile onset of tics in $D1$ transgenic mice, as well as alleviation of tics after administration of clonidine, a drug effective in the treatment of TS [123].

Inhibitory GABAergic neurons project directly from the striatum to the GP interna and externa. A decrease in GABAergic projection might cause decreased inhibition of excitatory thalamocortical neurons, leading to increased glutamatergic cortical excitation and the expression of tic behavior. Alternatively, reduced activity of cerebral cortex GABAergic interneurons arising from the same embryonic regions as the GABAergic striatal projection neurons [124, 125] may play a role in tic behavior, for example, through a loss of neurons in the striatal matrix compartment during a specific point in development [125]. Evidence for the involvement of NE in TS stems from the therapeutic effects of the presynaptic α_2 receptor agonist clonidine as well as observations that TS symptoms can worsen during stress, in which NE plays a significant role. Compared to controls, TS subjects had higher stress levels and urinary NE levels prior to undergoing lumbar puncture, while these findings were significantly correlated to clinician ratings of tic severity [59]. Another study found NE levels in the CSF to be 55% higher in TS subjects than in controls [61], while CSF corticotropin-releasing factor was found to be higher in TS compared to OCD and normal control subjects [60]. These findings support involvement of NE in TS. Preliminary data indicate that genetic factors are not involved with the role of NE in TS, as evidenced by a study assessing possible differences in the NE transporter gene (*NET*) between TS and control subjects, which found no mutations of functional significance [126].

Opioids are concentrated in the basal ganglia and are known to interact with central dopaminergic neurons. Postmortem studies [127] indicated decreased levels of dynorphin A immunoreactivity, while a CSF study [128] showed elevated concentrations of dynorphin A (1–8) in CSF of TS patients, suggesting a role for endogenous opioids in TS. Although the latter finding was not replicated in a follow-up study [129], further evidence comes from a small dose–response study [130] showing a significant decrease

in postdrug tic frequency following low-dose but an increase after intermediate-dose spiradoline, a κ -opioid agonist. Similar dose–response findings were found when TS subjects were challenged with naloxone, an opiate antagonist, indicating a decrease in tics following low-dose naloxone, while a high dose resulted in a tic increase [131]. Therefore, the influence of opioids on TS may be based on a dose–response effect.

10.8.3 Second-Messenger Systems

Involvement of second-messenger systems, in particular a postreceptor defect, has also been proposed as a contributing factor to abnormalities in neurotransmitter systems involved with TS [108, 132], especially since many neurotransmitters interact with cyclic adenosine-3′5′-monophosphate (cAMP); adenylyclase is activated by α_2 receptors; while opiate, α_2 -adrenergic, D_2 , serotonergic, and muscarinic receptors inhibit cyclase activity. This hypothesis was supported by a postmortem study of three adult TS subjects [108] which found a 34–56% cAMP reduction in frontal, temporal, and occipital cortices and a 23% reduction in the putamen. The study indicated dopaminergic abnormality in TS but suggested that the mechanism involved significant alteration at the DA uptake sites. A follow-up study, however, did not confirm significant alterations in second-messenger systems in either cortical regions or blood in TS subjects.

10.9 TREATMENT

10.9.1 Introduction

Treatment of TS requires a biopsychosocial approach [133, 134]. A good understanding of the natural history of TS is important for the choice of treatment. Upon examination of the patient with TS, the full range of difficulties and competencies should be charted, while onset of tic symptoms, progression, waxing and waning, and factors that worsened or ameliorated tic status should be assessed [134]. Educating patients and their families about the course of the TS is the first and most important step in treatment [135]. A lack of understanding of the illness may itself be a cause of significant stress. Acute and chronic stress can exacerbate tics, and psychotherapeutic interventions may enhance adjustment, self-esteem, and family relations [136]. In uncomplicated cases of TS psychosocial management may successfully manage the symptoms without pharmacological intervention. If comorbid disorders such as OCD or ADHD are present, successful treatment of these disorders may lead to alleviation of tic severity.

Due to the natural waxing and waning pattern of tics and the ability of patients to suppress symptoms, clinical decision making about initiating medication and dose adjustment can be a challenge, in particular since most tic-suppressant drugs take several weeks to reach their full effect, leading to difficulty discerning between drug response and spontaneous waning of symptoms.

10.9.2 Neuroleptics

The typical neuroleptics have been best studied in TS [137], with the first description of successful treatment with haloperidol reported in 1961 [138], while haloperidol and

pimozide have been most extensively studied in controlled or head-to-head trials [102, 137]. Compared to current practice, older studies used higher dosage ranges [102], and due to the difference in potency, most studies used, for example, higher doses of haloperidol compared to pimozide. However, in a more recent numerically comparable crossover study of haloperidol and pimozide [139], pimozide was found to be superior to haloperidol, whereas haloperidol was not more effective than placebo. Interestingly, the latter was concluded after assessing symptom change by self-report, while clinician ratings showed some improvement of symptoms. Haloperidol did cause a threefold higher rate of dose-limiting side effects such as extrapyramidal symptoms. The latter may explain the results of a 15-year follow-up study [140] indicating that over the long term significantly more patients are likely to remain on pimozide (12 of 13) than haloperidol (9 of 17). Recent evidence shows that at about 2 mg per day haloperidol occupies 80% of the dopamine receptors [141], providing support for the fact that lower dosages appear sufficient in most TS patients. Fluphenazine and trifluoperazine, two other typicals, have also been used to treat TS [142–145]. Both were found to be as efficacious as haloperidol but caused fewer side effects.

The use of typical neuroleptics in TS has diminished due to the burdensome side effects of these medications—in particular, extrapyramidal symptoms [146, 147] and pimozide-induced QT prolongation. In addition, cytochrome P450 (CYP450)–induced interactions with other medications may lead to decreased efficacy versus increased serum levels, with subsequent worsening side effects or potential toxic levels of medications and even fatal results [148, 149]. A rare case of fatal impaired drug metabolism in a TS patient due to genetic polymorphism of CYP450 loci has been reported [150].

Rebound symptom exacerbation after decrease or discontinuation of pharmacological treatment, response latency, the fluctuating course of tics, and side effects such as sedation all support starting treatment with a low dose and making slow up- and downward adjustments [137]. See Table 10.2 for recommended dosages.

Development of the atypical neuroleptics, combined serotonergic and dopaminergic antagonists, has led to gradual replacement of the typical neuroleptics as the first line of treatment, since they carry an apparently lower risk of extrapyramidal symptoms such as dystonias and akathisia but have equal treatment efficacy [137]. In particular, risperidone has been studied in TS. Several open-label [151, 152] and placebo-controlled studies [153, 154] as well as comparison studies of clonidine [155] and pimozide [156] versus risperidone showed significant improvement of tics, while clonidine and pimozide were found to be as effective as risperidone. In subjects with TS and comorbid OCD, improvement of OCD symptoms was observed as well. Most common side effects were sedation, weight gain (in particular in patients younger than 18 [156]), increased appetite, and mild extrapyramidal symptoms. The latter was observed twice as often in the pimozide compared to the risperidone group. Depression was also reported as a side effect of risperidone. A retrospective study evaluating this phenomenon in 58 adult and adolescent TS subjects treated with risperidone [157] found that 17 subjects (29.3%), 9 of whom had a previous history of depression, developed major depressive disorder, including 1 who later committed suicide, while 13 (22.4%) developed dysphoria. Twenty-one (70%) of these patients discontinued the use of risperidone because of this side effect.

TABLE 10.2 Medications Used to Treat TS

Medication	Starting Dose (mg/day)	Range (mg/day)	Side Effects
<i>Neuroleptics</i>			
Haloperidol	0.25	0.25–2.5	Weight gain, sedation, dystonia, akathisia, dry mouth, blurred vision
Pimozide	0.5	0.5–4	Electrocardiographic (ECG) changes, dystonia, akathisia, weight gain
Risperidone	0.5	0.5–3	Weight gain, sedation, depressed mood, dystonia, hyperlipidemia
Olanzapine	2.5	2.5–10	Weight gain, sedation, hyperlipidemia, glucose metabolism disturbance
Ziprasidone	5	5–40	Sedation, ECG changes
Sulpiride ^a	200	200–1000	Sedation, weight gain, akathisia, depression
Tiapride ^a	4 mg/kg	6 mg/kg	Nausea
<i>α₂ Agonists</i>			
Clonidine	0.05	0.05–0.3	Sedation, dizziness
Guanfacine	0.5	0.5–3	Mild sedation

^aNot available in the United States.

Olanzapine has been studied in adults in a few, small, open-label studies [158, 159] and in a 52-week crossover study with pimozide involving 4 patients [160]. Its effectiveness on aggressive behavior and tic severity was also assessed in a single-blind study involving 10 children ages 7–13 [161]. These studies showed that olanzapine can significantly decrease tic severity; however, as with risperidone, sedation and weight gain were found to be the most common side effects.

The effects of ziprasidone in TS have only been described in one double-blind placebo-controlled study [162] showing significant reduction in tic frequencies compared to placebo. A recent case report [163] described equal effectiveness of tiapride, a neuroleptic not available in the United States, and ziprasidone. However, a switch to ziprasidone was made due to significant tiapride-induced weight gain, all of which was lost while the patient was treated with ziprasidone.

Significant tic reduction was reported with the use of quetiapine in a small open-label trial with sedation as the main side effect. Further beneficial effects are reported in several case reports [164–166]. The use of aripiprazole, the latest atypical introduced in the United States, has not been reported yet.

Significant weight gain has been reported as a common side effect in typical neuroleptics as well as in the atypicals olanzapine and risperdal, with moderate weight gain and weight neutrality respectively in quetiapine and ziprasidone. Neuroleptics-induced weight gain can lead to disturbance of glucose and lipid metabolism and subsequent diabetic ketoacidosis or cardiovascular disease [167–169]. However, neuroleptics may also alter glucose metabolism independent of weight gain [169].

These are important factors in considering the use of atypicals in TS. Compared to the other atypicals, ziprasidone appears to have a greater propensity to increase the QT interval. To date, no fatalities have been reported. However, it should be used with caution in combination with other medications that can prolong the QT interval.

10.9.3 Other Antidopaminergic Agents

Tetrabenazine is a weak postsynaptic DA antagonist and reuptake inhibitor. Not yet available in the United States, its moderate success in improving tic behavior was reported in 11 of 17 TS patients treated with the drug [144].

Tiapride, sulpiride, and amisulpiride are selective D₂ blockers unavailable in the United States, but commonly used for treatment of TS in Europe. Tiapride was superior to placebo in the treatment of tics in a double-blind crossover study involving 27 children with TS [170]. The use of sulpiride has been reported in a retrospective study of 63 TS subjects [171], indicating improvement of symptoms in 60% of patients, while amisulpiride was reportedly effective for tic suppression in 2 cases of TS [172]. Most common side effects for tiapride and sulpiride were sedation and weight gain.

Pergolide is a mixed D₂-D₁ agonist believed to reduce dopaminergic transmission. In an open-label study 75% (24 of 32) of children with TS reported at least a 50% reduction in tic severity. A recent placebo-controlled study including 57 children and adolescents ages 7–19 randomized 2:1 to pergolide or placebo showed that, compared to placebo, pergolide treatment was associated with lower tic severity scores, while in subjects with TS and comorbid ADHD, improvement of ADHD symptoms was observed as well. Given the high comorbidity of ADHD and TS, the latter is of interest if confirmed in other studies, since it would be useful to have a single medication that can improve both ADHD and TS symptoms. Most common side effects of pergolide were mild gastrointestinal problems and mild sedation.

10.9.4 The α_2 Agonists

The use of the centrally acting α_2 -adrenergic antihypertensive drugs clonidine and guanfacine in TS is based on the hypothesis that tics are exacerbated by stress and the fact that some TS patients do not respond to haloperidol [173]. To date, studies investigating the use of clonidine in TS have been contradictory. Initial open-label studies showed mixed results [173, 174] while a 60-week, small, placebo-controlled study showed significant symptom improvement in only 6 of 13 subjects [175]. A large, open-label study comparing clonidine with neuroleptics showed greater symptom improvement with neuroleptics compared to clonidine, while only a small number of subjects seemed to have benefited from clonidine [176]. A 6-month placebo-controlled clonidine crossover study in 30 subjects with TS found that the use of clonidine did not significantly reduce tics [177]. Results of double-blind studies were also mixed: One study found clonidine to be equally effective as risperidone [155] while another found minimal tic reduction [178]. Comparison studies in TS subjects with TS and comorbid ADHD found that both methylphenidate and clonidine reduced tics [179], while a study comparing desipramine and clonidine found no effect of clonidine on tics. Furthermore, a paradoxical increased tic response to clonidine has been reported [180, 181]. These findings show that clonidine

may reduce tics as well as hyperactivity. The most common side effect was daytime sedation [179].

Guanfacine has a longer half-life than clonidine, requiring fewer daily dosages, and is less sedating than clonidine, making it a more tolerable alternative to clonidine. However, no comparison study exists testing this assumption. As with clonidine, the results of guanfacine studies in TS are mixed: One small, open-label [182] and a larger, double-blind, placebo-controlled study [183] found a significant decrease in tics, but an equally large, placebo-controlled study found no benefits of guanfacine. A case report describing new-onset manic symptoms in a patient recently started on guanfacine suggests that guanfacine may induce mania in vulnerable subjects [184].

10.9.5 Other Tic Suppressants

Baclofen was studied in a large, nonrandomized comparison study with botulinum toxin type A (Botox) [185], showing a significant decrease in symptoms in 250 of 264 subjects. However, a small, double-blind, placebo-controlled study found only reduction in the impairment caused by tics rather than a tic reduction [186]. Several studies indicate that Botox alone causes significant tic reduction when injected at the site of their most problematic tics—directly in the vocal cords in the case of phonic tics [187–189]. Up to 80% of subject also reported a decrease in premonitory urges. The most frequent side effect was hypophonia.

Nicotine chewing gum was found to substantially decrease tics in patients treated with haloperidol [190], a finding confirmed by several studies using nicotine dermal patches [191–193]. The most common side effect was nausea. One study reported nicotine-mediated tic improvement in unmedicated subjects. To date, this finding has not been replicated in a placebo-controlled study. It appears that nicotine can have an adjunct role in TS treatment. Open-label studies of mecamylamine, a nicotine antagonist, showed reduced tic severity in 22 of 24 subjects [194, 195]. However, a follow-up double-blind, placebo-controlled study in 61 TS subjects found no benefit of mecamylamine [196]. Finally, opioid receptor agonists [130] and antagonists [131] may have a dose-related moderate effect on tic suppression.

10.9.6 Nonpharmacological Treatments

Multiple behavioral interventions have been tried in the treatment of tics. However, to date no one particular behavior treatment has been proven to be successful. One promising treatment is habit reversal training (HRT), shown to be effective in two pilot randomized clinical trials [197, 198]. The components are awareness training and competing response training. In awareness training the patient is taught to identify the beginning of a tic or bout of tics and the situations in which they occur. Once identified, the patient is then taught to impose a voluntary competing movement incompatible with the tic. Two large-scale clinical trials, one in children and one in adults, are currently underway to assess the efficacy of HRT.

Neurosurgical procedures have been proven to be highly effective in patients with intractable TS [199]. In three adult patients unresponsive to any drug or alternative treatment, bilateral thalamic high-frequency stimulation was performed at the level of the centromedian nucleus substantia periventricularis and the nucleus ventro-oralis

internus. The treatment was without serious complications and led to complete, continued disappearance of symptoms. This treatment should only be considered in patients with severe, intractable TS persisting into adulthood.

10.10 FUTURE DIRECTIONS

Advances in the neurosciences have provided us with current ideas about TS. Future advances may provide us with identification of vulnerability genes, which in turn may lead to new therapeutic directions. However, the clinical course of TS starts in childhood and is affected by genetic, neurobiological, and environmental factors as well as by the presence of comorbid disorders. Animal models may be helpful in studying the interplay of these factors from a developmental perspective and not only help us better understand the cause of TS but also possibly predict the course and treatment response of TS. A recent development is the renewed interest in neuronal oscillations: the notion that cortical neurons form behavior-dependent oscillating networks. These oscillations bias input selection, temporarily linking neurons into assemblies, thus facilitating synaptic plasticity, mechanisms cooperatively supporting temporal representation and long-term consolidation of information [200]. TS may be the result of aberrant neuronal oscillations [201], and future advances in this area may also aid in a better understanding of the pathophysiology of TS as well as an understanding of the efficacy of treatment.

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11

NEUROPHARMACOLOGY OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

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11.1	Introduction	291
11.2	Rationale for Treatment	292
11.3	Evaluation and Diagnosis	292
11.4	Pharmacotherapy of ADHD	293
11.4.1	Stimulants	293
11.4.1.1	Methylphenidate Hydrochloride	294
11.4.1.2	Dexmethylphenidate Formulations	296
11.4.1.3	Amphetamines	296
11.4.1.4	Stimulant Adverse Effects	298
11.4.2	Nonstimulants: Primarily Noradrenergic Agents	299
11.4.2.1	Tricyclic Antidepressants	299
11.4.2.2	Atomoxetine	299
11.4.2.3	Clonidine and Guanfacine: α_2 Agonists	299
11.4.2.4	Bupropion	300
11.4.2.5	Venlafaxine	301
11.4.3	Miscellaneous Agents	301
11.4.3.1	Modafinil	301
11.4.3.2	Antipsychotic Agents	301
11.5	Summary and Reflections on Clinical Practice	301
	References	302

11.1 INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disorder characterized by dysfunctional levels of overactivity, inattention, and impulsivity

[1]. These behaviors are excessive compared to normative levels for age-matched peers with symptoms causing dysfunction in two or more settings. Variability of symptoms is increasingly recognized as a hallmark of the disorder, with inconsistency observable on a minute-by-minute, hourly, and daily basis. Although doubts have been raised about the validity of ADHD as a medical disorder, an incontrovertible body of data supports its neurobiological bases, with increasing evidence of continuity from preschool age through adulthood. The cross-cultural prevalence of ADHD is estimated to be within the range of 3–17% [2], with most estimates converging between 5 and 10% during elementary school. Pharmacotherapy prevalence of about 1% has been reported in preschool-aged children [3], and recent estimates place prevalence in adulthood at about 2–4% [4].

The current definition of ADHD in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR [5]) includes two subgroups of symptoms of hyperactivity/impulsivity and inattention. These symptom sets allow for two partial subtypes, when sufficient criteria (six of nine) are met for only one of the two categories, and for one combined type, when sufficient symptoms are present in both. The combined form is the most common in school-age children (60%), with the inattentive type accounting for most of the remaining population of ADHD (~35%) [6].

11.2 RATIONALE FOR TREATMENT

The diagnosis of ADHD requires that the symptoms be associated with impairment in educational, occupational, or social functioning. Treatment is instituted with the implicit goals of arresting and reversing those impairments. However, there are no standard criteria for defining impairment, which admittedly would constitute a major challenge in order to cover the life span and the wide range of functioning that can be deleteriously affected by ADHD. Pragmatically, impairment during the elementary school years is referenced to parent and teacher expectations, and outcome measures are defined in terms of improvement on rating scales that quantify those expectations. Based on this framework, efficacy of pharmacological and nonpharmacological treatments has been definitively established in short-term usage (days to weeks) and strongly suggested in medium-term usage (months to over one year). However, there are no data sets that can conclusively demonstrate the long-term benefits of treatment, and given the evolution of ethical standards, there is no prospect of definitive studies ever being conducted with currently available treatments, at least for ADHD as it is currently conceived.

Despite such uncertainties, the short-term benefits of treatment, particularly with stimulants, can be so robust that they provide their own nearly irresistible rationale [7, 8]. The few naturalistic longitudinal studies suggest that untreated ADHD can lead to malignant long-term outcomes, including conduct disorder, substance abuse and dependence, and antisocial personality disorder [9–16]. Even when the outcomes are less severe, educational and occupational underachievement is common.

11.3 EVALUATION AND DIAGNOSIS

Treatment of ADHD begins with a careful and thorough diagnostic process to rule out alternative explanations for the presenting symptoms as well as to establish any

comorbid diagnoses that may guide the choice of therapy. However, the commonality of the disorder, the scarcity of clinician time, the low reimbursement patterns for pediatricians, and the absence of objective diagnostic tests combine to produce a diagnostic process that can appear rushed and imprecise. Fortunately, much information is now available through support organizations and on the Internet, and many parents avail themselves of these sources prior to consulting a clinician. For example, Google lists over 12,000,000 pages on ADHD as of this writing, leading with the authoritative site www.chadd.org.

A diagnostic evaluation begins by establishing the reliability of the information sources (optimally including a primary teacher's written or telephone report) and documenting the presence of sufficient impairing symptoms in more than one setting. Alternative explanations must also be considered, including acute or chronic trauma or abuse. Associated comorbid conditions, including learning disorders, other disruptive behavior disorders, and Tourette's disorder, must also be considered. Other common psychiatric diagnoses, including anxiety and mood disorders, must also be excluded. Asperger's syndrome can be missed, particularly in children with above-average general intelligence and attentional problems. Similarly, psychotic disorders must be considered as alternative causes of hyperactivity and cognitive/attentional impairments in adolescents or adults. One of the key elements of the assessment process should be the compilation of an inventory of the strengths and resources available to the patient. This phase is often overlooked in the process of documenting the extent of pathology, but it is crucial for both short- and long-term planning and for formulating the prognosis.

Once the diagnosis is established, most patients with ADHD usually require both pharmacological and supportive treatments. Before elaborating on pharmacotherapy, we briefly mention some of the supportive services important in ADHD. Psychoeducation regarding the diagnosis, its neurobiological roots, and the complex interplay between environmental support and genetic/temperamental factors within the framework of developmental maturation provides an essential foundation for the long-term therapeutic alliance that must be established. Psychosocial treatments such as behavioral modification plans, token economies, frequent reminders to stay on task, and positive reward systems have high parental acceptability but are time consuming, difficult to maintain over long intervals, and inferior to optimally administered medication treatment for the majority of school-age children with ADHD [17–19]. The combination of optimal medication and psychosocial treatments can improve outcome in highly disadvantaged children, can reduce the dose of medication required to achieve equivalent benefits, and may be sufficient in milder cases who also have a high ratio of supports and resources to deficits and risks [20].

11.4 PHARMACOTHERAPY OF ADHD

11.4.1 Stimulants

The extraordinary efficacy of psychostimulants for the short-term amelioration of hyperactivity and inattention was first reported in 1937 by Charles Bradley [21], who was searching for a pharmacological method of treating post-lumbar puncture headache [22]. Bradley's description of the effects of benzedrine (racemic

amphetamine) and his thoughtful speculations regarding the mechanism of action remain so current nearly 70 years later that his initial report was recently reprinted in a book on the basic and clinical neuroscience of stimulants in ADHD [1]. Nevertheless, pharmacological treatment for psychiatric or behavioral symptoms did not fit the dominant psychoanalytical *zeitgeist* during the mid-twentieth century [23]. The less cardiovascularly active stimulant methylphenidate was patented in 1954 [24]; its use as a treatment for “minimal brain dysfunction” [25], hyperactive child syndrome, and eventually DSM-III attention-deficit disorder with or without hyperactivity [26] began in the 1960s.

11.4.1.1 Methylphenidate Hydrochloride. Methylphenidate hydrochloride, commonly known by its original brand name, Ritalin, remains the most prescribed stimulant for ADHD under a wide range of trade names and as a generic product (see Tables 11.1, 11.2). Methylphenidate inhibits noradrenergic and dopaminergic reuptake through the respective synaptic transporters. In vivo functional neuroimaging studies document that clinically relevant doses of methylphenidate bind the majority of available striatal dopamine transporters (DATs) [27] and that the net result is an increase in synaptically available neurotransmitters, but only in response to intrinsic neuronal activity. Thus, methylphenidate enhances ongoing neurochemical modulation at catecholaminergic synapses.

The pharmacokinetics of methylphenidate have been thoroughly studied [28–37]. Methylphenidate is rapidly absorbed from the gastrointestinal tract with substantial first-pass elimination. The active parent compound is metabolized in the liver, and the inactive metabolites are excreted renally. Methylphenidate exists in four stereoisomeric forms: standard methylphenidate compounds contain racemic D, L-*threo*-methylphenidate, without the erythro form [34, 38–40]. Although both D- and L-methylphenidate are present in equal proportions, L-methylphenidate is converted more rapidly to inactive L-ritalinic acid [34], resulting in a preponderance of D-methylphenidate in blood. The excretion half-life of D-methylphenidate averages about 3 h (range 2–4.5 h) [29, 36, 41], which results in essentially total clearance (~5 half-lives) of active drug from plasma by the next day. Studies of the relationship

TABLE 11.1 Immediate-Release Stimulants

Drug	Dose (mg)	Typical Duration (h)	Schedule
Methylphenidate (Ritalin, Metadate, Methylin, and generic)	5, 10, 20	3–4 h	b.i.d. to t.i.d.
Dexmethylphenidate (Focalin)	2.5, 5, 10	3–4 h	b.i.d. to t.i.d.
Dextroamphetamine (Dexedrine and generic)	5, 10	4–8 h	q.d. to b.i.d.
D/L-Amphetamine (Adderall and generic)	5, 7.5, 10, 12.5, 15, 20, 30	4–8 h	q.d. to b.i.d.

TABLE 11.2 Extended-Release Methylphenidate

Drug and Dose Available	Method	Immediate Release	Extended Release	Typical Duration (h)
Ritalin SR, 20 mg; Metadate SR, 10 and 20 mg	100% wax matrix	Minimal	More sustained than immediate release	5
Concerta ER, 18, 27, 36, and 54 mg	Osmotic system	22% overcoat	78%	10–12
Metadate CD, 20 mg	Beads	30%	70%	8
Ritalin LA, 20, 30, and 40 mg	Beads	~ 50%	~ 50%	8
Focalin XR, 5, 10, and 20 mg	Beads	~ 50%	~ 50%	8

between plasma drug level and efficacy led to the suggestion that escalating blood levels are required to compensate for hypothesized short-term tachyphylaxis. An osmotically activated extended-release formulation (trademark OROS, brand name Concerta) was designed to produce steadily increasing blood levels for up to 10 h [37, 42, 43]. The commercial success of this approach has been substantial, although the proliferation of alternative effective formulations with differing pharmacokinetic profiles, including a transdermal patch that delivers nearly constant blood levels [44–46], weakens the theoretical argument for the necessity of escalating plasma levels.

The short half-life of methylphenidate means that immediate-release preparations need to be administered multiple times per day. Prior to the dissemination of the results of the National Institute of Mental Health (NIMH) multimodal treatment study of ADHD (MTA), the standard of care was to provide stimulant treatment during the hours of the school day, generally by administering doses after breakfast and after lunch at school. This pattern was typical of the treatment provided to children in the MTA study who were randomized to receive usual treatment (referred to as community care). Children randomized to receive medication through the MTA study were given three daily doses of immediate-release stimulant, generally methylphenidate, so as to extend medication effects into the after-school hours to facilitate homework and minimize social impairments. Although the MTA study did not include a placebo control group, the children assigned to community care improved less than any of the other three treatment groups and were particularly less improved compared to those who had been assigned to treatments that included medication. This dramatic difference in outcomes at the 14-month follow-up coincided with the increasing availability of extended-release formulations of methylphenidate as well as amphetamines, which have intrinsically longer durations of action.

In the United States, extended-release preparations, particularly containing methylphenidate, are now usually prescribed as first-line therapies for ADHD. The substantially increased cost, relative to immediate-release generics, is compensated by the greater ease and increased likelihood of adherence, since one morning dose can replace up to three doses of immediate-release medication. Other advantages include

TABLE 11.3 Extended-Release Amphetamines

Drug and Dose Available	Method	Immediate Release	Extended Release	Typical Duration (h)	Comments
Dexedrine Spansules, 5, 10, and 15 mg	Beads	~ 1/3	~ 2/3	8–13	Excretion $T_{1/2} > 10$ h for all amphetamines
Adderall XR, 5, 10, 15, 20, 25, and 30 mg	Beads	~ 50%	~ 50%	12 +	Absorption may be delayed by high-fat breakfast

protection of confidentiality, decreased concern regarding the stigma of standing outside the school nurse's office or being called to the health office or the principal's office over the public address system, and decreased likelihood of illegal diversion. The theoretical benefits of fewer rapid fluctuations in the brain levels of methylphenidate and the more gradual daily discontinuation can also minimize the rapid return of symptoms referred to as "rebound" [47].

All extended-release methylphenidate formulations that are currently approved by the U.S. Food and Drug Administration (FDA) contain D,L-methylphenidate, although they differ in method of extending the absorption phase and intended duration. The OROS method used by Concerta was the first of the new generation extended-release formulations. It is designed to deliver 22% of its total dose as an immediate-release overcoat and to provide a steadily ascending blood level over the next 9–10 h so as to produce up to 12 h of symptom reduction compared to placebo [43, 48]. Metadate CD and Ritalin LA both use beads that are absorbed immediately or after a delay of about 4 hours to extend their efficacy to about 8 h. They differ in the proportion of immediate- to delayed-release methylphenidate: Metadate CD is designed to release about 30% of methylphenidate immediately and 70% delayed [49–51], whereas Ritalin LA contains equal proportions for immediate and delayed delivery.

11.4.1.2 *Dexmethylphenidate Formulations.* The more active dextro isomer of methylphenidate is now also marketed as immediate-release (Focalin) [52, 53] and in an extended-release formulation (Focalin-XR) [54]. Placebo-controlled clinical trials have demonstrated efficacy for ADHD symptoms [52, 53]. Because the relatively inactive levo isomer is not included, the dose administered is half of the racemic dose. One study compared dexmethylphenidate and racemic methylphenidate against placebo control and found equivalent efficacy (effect size 1.0 for each) for both formulations, with some evidence of greater duration of action for dexmethylphenidate [53]. Controlled comparisons of Focalin-XR to other extended-release formulations of racemic methylphenidate or to amphetamines have not yet been conducted.

11.4.1.3 *Amphetamines.* The sympathomimetic effects of amphetamines have been known since racemic amphetamine (benzedrine) was first synthesized in 1936 [21, 23].

Amphetamines are currently available in a wide range of immediate and extended-release receptor products (see Tables 11.1 and 11.3). By contrast to methylphenidate, which is a pure catecholaminergic transport reuptake inhibitor, amphetamines function at multiple molecular levels. Comparisons of the dextro and levo isomers led to the conclusion that the dextro isomer, D-amphetamine, is more robust in promoting presynaptic dopamine (DA) and norepinephrine (NE) release than levoamphetamine [55–61], and the suggestion that dextroamphetamine is somewhat more efficacious [62], although the behavioral effects of levoamphetamine in rat are longer than those of dextroamphetamine [60]. Amphetamines differ from methylphenidate in at least three ways. First, they release monoamines through a calcium-independent mechanism [63, 64], presumably by converting DATs and NE transporters (NETs), respectively into channels that allow the massive efflux of thousands of molecules per second instead of recycling one molecule of DA or NE per second [65]. This profound increase in synaptic catecholamines is independent of neuronal activity and is far greater quantitatively than levels obtainable with reuptake inhibitors such as methylphenidate or cocaine. Second, amphetamines reverse flow at the vesicular monoamine transporter (VMAT2), thus also increasing synaptic levels of serotonin (5-hydroxytryptamine, or 5-HT). Through a third mechanism, amphetamines inhibit the catabolic enzyme monoamine oxidase (MAO) [66], which also increases synaptic levels of all three monoamines (DA, NE, 5-HT). In sum, amphetamines produce markedly greater synaptic concentrations of monoamines than methylphenidate, these effects are independent of endogenous neuronal activity, unlike those of methylphenidate, and amphetamines produce fairly substantial serotonergic effects, again, unlike methylphenidate. Thus, the substantial therapeutic equivalence of the two classes of stimulants [67–71] represents an unsolved puzzle. Currently, the major clinical differentiation between amphetamines and methylphenidate compounds is based on pharmacokinetic differences, which are substantial, with amphetamines averaging excretion half-lives of 10 h or more, versus about 3 h for methylphenidate [41, 72–74]. Such major differences result in longer durations of action for even immediate-release amphetamines, which also extend the duration and potential severity of adverse effects [75, 76].

The potential differences between the levo- and dextroamphetamine isomers and the possibility that they may combine synergistically has been exploited in a preparation of four salts that has had a major impact commercially and clinically under the trade name Adderall [77–82]. Although relatively few studies have contrasted levo- and dextroamphetamine, some reports suggest possible differences that may have clinical utility.

Low-dose levoamphetamine decreases locomotion in mice while increasing activity at higher doses [83]. In rat hippocampus, dextroamphetamine evokes a faster and more intense release of dopamine [84]. Dextro- and levoamphetamine treat distinct symptoms in a canine model of narcolepsy [85]. A recent independently funded study found that racemic amphetamine releases DA with a faster onset and offset than D-amphetamine alone when the drugs are locally applied to the striatum and nucleus accumbens core of anesthetized rats [86]. The amplitude of DA release was highly correlated with the relative amount of D-amphetamine that was locally applied and the addition of L-amphetamine only altered the time course of DA release. When L-amphetamine was applied alone, it was able to elicit DA responses with an amplitude slightly lower than that of D-amphetamine alone. These findings

are consistent with reports of differential potency and effect of levoamphetamine and dextroamphetamine in humans [61] as well as basic science studies in laboratory animals [87–89]. In transfected human embryonic kidney (HEK) 293 cells, levoamphetamine interacts with the human DAT [90] with nearly identical transporter currents, although its potency is 3–6 times lower than that of dextroamphetamine. The recent findings for levoamphetamine [86] do differ from studies that failed to find kinetic differences [84, 85], but this is the first time these comparisons have been carried out using the temporal resolution of voltammetry and with rapid, first-time exposure to stimulants rather than the more prolonged exposures of systemic administration or reverse microdialysis. However, contributions of the various effects of dextroamphetamine and levoamphetamine, whether alone or in combination, and the relevance of these interactions at therapeutic concentrations in children with ADHD have not been elucidated. In a head-to-head double-blind, placebo-controlled, independently funded comparison between immediate-release Adderall and immediate- and delayed-release formulations of dextroamphetamine, 76% of children were discharged on one of the dextroamphetamine formulations, and only 24% of participants were judged to have obtained their best effects on Adderall [91]. The extended-release formulation of Adderall was not included in that trial [91], as it had not yet been marketed.

11.4.1.4 Stimulant Adverse Effects. Relatively mild adverse effects of stimulants are common and particularly result in decreased appetite and delayed onset of sleep. These are related to the dose of a given stimulant and, on average, are more intense with delayed-release preparations and with the amphetamines than with methylphenidate-based compounds [92]. The anorexic effects of the stimulants have raised questions regarding long-term effects on growth, particularly height. Most studies concluded that long-term effects were negligible or slight [93–105]. However, the pattern of medication use has changed over the past decade from an earlier tendency to medicate twice per day with immediate-release methylphenidate primarily during school days to all-day coverage through the use of extended-release formulations that are administered daily throughout the year [106]. Effects on growth suppression are expected to be more pronounced on amphetamines, but even with methylphenidate, there is some evidence of mild growth suppression which can be marked in some individuals [107, 108].

Because stimulants are prescribed chronically during a period of continuing brain development, the potential for less frequent but enduring or severe adverse effects must always be considered. The worry that stimulants might be etiologically responsible for triggering Tourette's syndrome has largely been resolved in the negative [109], although there is a complex relationship between stimulant type, dose, and tic frequency/severity [110]. The possibility that stimulants would produce enduring changes in brain circuits that would predispose individuals to later drug abuse has also not been supported by the available literature, although such evidence is invariably flawed [111] since placebo-controlled long-term studies cannot be conducted ethically. Rare reports of psychosis [76] or cerebral vasculitis [112, 113] highlight that all medications carry risks, but on the whole, the stimulants are remarkably safe with respect to the hepatic, hematopoietic, cardiovascular, and gastrointestinal systems [114].

11.4.2 Nonstimulants: Primarily Noradrenergic Agents

11.4.2.1 Tricyclic Antidepressants. The tricyclic antidepressants (TCAs) have long been available as second-line drugs for the treatment of ADHD [115–120], although their use has decreased dramatically in recent years because of concerns about potentially lethal cardiovascular effects, particularly with the highly noradrenergic TCA desipramine [121–123]. Another limiting factor has been the high prevalence of adverse effects such as constipation, tachycardia, and dry mouth [124–126].

Clomipramine and desipramine in doses of up to 3.5 mg/kg/day were found to be superior to placebo for ADHD symptoms but less efficacious than methylphenidate [127]. In direct comparison studies, stimulants have proven to be superior to TCAs [120]. Although TCAs remain a reasonable option for treating ADHD, especially when comorbid with enuresis [128], or Tourette's disorder [129], they have largely been supplanted by the novel agent atomoxetine.

11.4.2.2 Atomoxetine. Atomoxetine is a specific NET reuptake inhibitor developed as a treatment for ADHD across the age spectrum [130, 131]. Since frontal DA is primarily deactivated by NET instead of DAT, atomoxetine increases levels of NE and DA in rat frontal cortex but not in the nucleus accumbens [132]. This regional selectivity likely underlies atomoxetine's absence of addictive liability which has made it the first FDA-approved medication for ADHD in children as well as adults without having to be classified by the Drug Enforcement Agency. In a large number of controlled trials supported by the manufacturer, atomoxetine has been found to be efficacious relative to placebo for hyperactivity/impulsivity as well as inattention symptoms [133–143]. In one open trial also supported by the manufacturer, atomoxetine was found to be comparable to methylphenidate [144]. Perhaps not surprisingly, when compared to stimulants in trials funded by the stimulant manufacturers, atomoxetine has been found to be less efficacious [145, 146]. In general clinical practice, the emerging consensus is that although atomoxetine is undoubtedly efficacious, its effects are typically less robust than the stimulant alternatives. Nevertheless, it has become a first-line treatment for ADHD when substance abuse concerns are in the forefront [147–149].

Despite its status as a nonstimulant, the common adverse effects of atomoxetine include anorexic effects on appetite, sleep difficulties, and gastrointestinal discomfort [150, 151]. These are generally mild, dose related, and typically self-limiting. Infrequent cases of more severe adverse events have been reported, including hypertension [152], initiation of mania [153, 154], seizures, and prolonged QTc after overdose [155].

11.4.2.3 Clonidine and Guanfacine: α_2 Agonists. The efficacy of clonidine, an α_2 agonist, was suggested by a pair of small controlled trials in ADHD [156, 157]. A pilot comparative trial without placebo control reported equivalent pre-post improvements for clonidine, methylphenidate, or the combination of both medications in children with ADHD and comorbid aggressive oppositional defiant disorder or conduct disorder [158]. In a double-blind crossover trial of clonidine, desipramine, and placebo in children with ADHD and Tourette's disorder, clonidine did not result in any significant improvements, in contrast with desipramine, which improved tic ratings and ADHD symptoms [159]. The only large randomized multisite placebo-

controlled comparison of clonidine with or without methylphenidate was conducted to determine its efficacy for the treatment of ADHD and comorbid Tourette's disorder [160]. Clonidine alone was found to be significantly more effective than placebo, but the combination of clonidine and methylphenidate was superior to either medication by itself for ADHD symptoms. In general, clonidine is tried when stimulants have been ineffective or when they might produce or have produced unacceptable adverse effects, whether on tic frequency and severity, sleep, or rebound return of symptoms. Despite the absence of controlled trials, the use of adjunctive clonidine for ameliorating sleep difficulties has been advocated by several authors based on case series and chart reviews [161–164].

Clonidine can be administered orally or as a transdermal patch. The lowest dose tablet is 0.1 mg, and treatment is usually started with fractional tablets (one-fourth or one-half) administered several times per day because of the short half-life (<4 h). Parents must be cautioned against abrupt discontinuation, because of the risk of rebound return of symptoms and even of hypertension [165–168]. The transdermal patch has the advantage of constant blood levels [169, 170], but the majority of children are reported to develop rashes in the locations where the patches have been placed [171]. Great care must be exercised with disposal of used patches [172], which typically need to be changed more frequently than the rate of once per week described for blood pressure regulation.

Clonidine is nonspecific in its agonist activity at all three α_2 receptor subtypes (A, B, and C) and perhaps at imidazoline receptors [173]. Guanfacine, on the other hand, is selective for the α_{2A} receptor [174–178]. The α_{2A} receptors are predominately found in the prefrontal cortex; guanfacine's decreased activity at α_{2B} receptors in thalamus and α_{2C} receptors in the brain stem may explain its decreased tendency to cause sedation [176]. Despite a strong theoretical rationale derived from extensive basic neuroscience observations [174–190], the only placebo-controlled double-blind trial of guanfacine was conducted for the treatment of ADHD symptoms in comorbid Tourette's syndrome, in which it was found efficacious for both types of symptoms [191].

The lack of patent protection for either clonidine or guanfacine in the United States has effectively prevented pharmaceutical company investment in exploring further uses for these agents, which are marketed as antihypertensives. The evidence of decreases in stress-induced distractibility in nonhuman primates amassed by Arnsten and colleagues suggests the potential benefit of adjunctive guanfacine in patients with ADHD who experience stress-related impairments in self-regulation [192].

Guanfacine has a longer half-life (17 h in adults) which minimizes rebound effects even when it is discontinued abruptly [193]. Usual doses begin at 0.5 mg once or twice per day, with titration to effect at about weekly intervals. Fatigue, sedation, and constipation can be observed but are usually mild and dose related. In children with familial risk factors for bipolar disorder, mania, or hypomania, symptoms have been described following initiation of guanfacine [194]. Increased valproate concentrations have also been described when coadministered with guanfacine [195].

11.4.2.4 Bupropion. Bupropion is primarily an inhibitor of noradrenergic reuptake, although it also has dopaminergic activity, as shown in animal studies [196–201]. The efficacy of bupropion for ADHD has been evaluated in several double-blind controlled trials [202–206] with modest effect sizes of lower magnitude than those available with stimulants in most studies and no statistical separation from placebo

in others [204, 207]. Bupropion in immediate-release formulation needs to be administered 2–3 times per day; the sustained-release formulation allows for twice-per-day administration. Divided doses are also recommended based on the recent finding that children and adolescents metabolize bupropion to active metabolites more rapidly than adults [208]. The principal serious adverse effect associated with bupropion is onset of seizures; one case report suggests that the combination of methylphenidate and bupropion at 300 mg per day may have been the cause of grand mal seizures in a 14-year-old [209]. Though fully controlled studies have not been conducted, open trials and single-blind studies suggest that bupropion may be effective for the treatment of ADHD combined with substance abuse disorders [210–212].

11.4.2.5 Venlafaxine. Multiple open trials have been conducted with venlafaxine for the treatment of ADHD [213–221], but the absence of double-blind, placebo-controlled trials and the relabeling by the FDA with a “black box warning” regarding the risk of suicidality in children and adolescents treated with antidepressants, including venlafaxine, have made this an even less commonly used option. The risk of hypertension makes monitoring vital signs essential.

11.4.3 Miscellaneous Agents

11.4.3.1 Modafinil. The wakefulness-promoting agent modafinil has been shown to be efficacious for ADHD in several double-blind, placebo-controlled trials [222–224]. Modafinil is pharmacologically distinct from all other validated treatments in having glutamatergic effects rather than direct effects on catecholamines [225–228]. Comparison-controlled studies are needed to determine the appropriate placement of modafinil in treatment algorithms for ADHD.

11.4.3.2 Antipsychotic Agents. Although not currently recommended as first- or second-line therapy for ADHD, typical antipsychotics were shown to be efficacious for treating ADHD in the 1970s [229, 230]. Their effectiveness for the behavioral manifestations of ADHD along with evidence that the typical neuroleptic haloperidol blocks the cognitive benefits of methylphenidate [231] suggests that multiple neural systems are involved in ADHD. The atypical neuroleptic risperidone is frequently used as an adjunctive treatment for ADHD in the presence of aggression or conduct disorder, but the only controlled trials for this compound indication are in children who also have below-average intelligence [232–234]. While the atypical neuroleptics were believed to have a lower risk of tardive dyskinesia, they are now acknowledged as being associated with a substantial risk of profound weight gain [235–239], vastly increasing the long-term probabilities of developing diabetes mellitus.

11.5 SUMMARY AND REFLECTIONS ON CLINICAL PRACTICE

The major finding of the MTA study was that standard treatment for ADHD in the community was surprisingly inferior to protocol-guided treatment [240]. That insight has yet to be universally embraced, but one of the means of improving care within

systems is to flexibly incorporate treatment algorithms [241, 242]. While a comprehensive review of such algorithms, which must be continuously updated, was not possible in this brief chapter, some of the basic principles of practice can be reiterated.

The *sine qua non* of treatment is still the clinician's judgment that the patient is experiencing substantial impairment from the symptoms of ADHD. Accordingly, treating pharmacologically is defensible even when the diagnosis must be qualified as ADHD—not otherwise specified, as long as the impairments are clearly and explicitly enumerated. The field now has a number of controlled studies that confirm the approximate dosage equivalence rules, such as the equivalence between 2 mg of methylphenidate and 1 mg of amphetamines or 1 mg of dexamethylphenidate. The MTA study has shown that determining if there is "room for improvement" with incremental dosages can be beneficial, but the general principle of "the best dose is the lowest" also must be respected. We now have many options available for the pharmacological treatment of ADHD, which is all to the good. On the other hand, there are few comparative studies, and most of those that have been funded by one manufacturer turn out to support that manufacturer's product. Thus, in the absence of substantial impartial comparative literature, the use of methylphenidate as the first agent in most cases is appropriate based on extensive experience and the demonstrated tendency to result in less severe adverse effects. At the same time, it is increasingly acceptable to start treatment with extended-release formulations rather than always beginning with immediate-release tablets.

For parents or families reluctant to embark on stimulant treatment, atomoxetine is a fully acceptable starting point. The unavoidable convenience of not being classified as a dangerous narcotic can compensate for the slower time to maximum benefit. Clinicians repeatedly fail to observe true equivalence of atomoxetine described by some controlled studies, but at the same time, there are few doubts that it is an efficacious compound. More work is needed to define the circumstances under which atomoxetine combined with a stimulant is most advantageous and when it may be risky. Certainly, such a combination makes regular monitoring even more important.

The combination of a stimulant and an α_2 agonist, particularly the longer duration agent guanfacine, has not been studied in controlled trials. Basic and clinical neuroscience findings and our own anecdotal experience suggest that this combination may be highly beneficial for those individuals who are highly stress responsive. Ironically, without guanfacine, sometimes such individuals can occasionally be worsened by stimulants, as predicted by studies of genetic variations in the catecholamine-*O*-methyltransferase gene [243]. A pharmacogenomics approach would be needed to test such a hypothesis, and we can anticipate that the tools of molecular genetics will increasingly become available to tailor our treatments, although that time is not yet here.

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12

PSYCHOPHARMACOLOGY OF AUTISM SPECTRUM DISORDERS

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12.1	Introduction	320
12.2	Pharmacological Studies In Autism Spectrum Disorders	321
12.2.1	Serotonergic Medications	321
12.2.1.1	Clomipramine	322
12.2.1.2	Fluvoxamine	322
12.2.1.3	Fluoxetine	323
12.2.1.4	Sertraline, Citalopram, Escitalopram, and Paroxetine	324
12.2.2	Dopaminergic Medications	325
12.2.2.1	Typical Antipsychotics	325
12.2.2.2	Atypical Antipsychotics	326
12.2.2.3	Stimulants	328
12.2.3	Miscellaneous Agents	330
12.2.3.1	α_2 -Adrenergic Agonists	330
12.2.3.2	Medications with Mixed Monoaminergic Mechanisms: Mirtazapine and Venlafaxine	331
12.2.3.3	Glutamatergic Medications	332
12.2.3.4	Antiepileptic Drugs	332
12.3	Targeting Syndromes within Autism Spectrum Disorders: Pragmatic Considerations	333
12.4	Conclusions	335
	Acknowledgements	335
	References	335

12.1 INTRODUCTION

The autism spectrum disorders include autistic disorder, Rett's disorder, childhood disintegrative disorder, Asperger's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) [1]. Collectively, these severe, enduring conditions are known as pervasive developmental disorders (PDDs). Autistic disorder is characterized by qualitative impairments in social interactions and communication and the presence of restricted and stereotyped behaviors, interests, and activities. Asperger's disorder is distinguished by nearly normal speech development and a lack of general intellectual impairment. Childhood disintegrative disorder is marked by a severe loss of acquired early skills in multiple areas after at least two years of apparently typical development. Rett's disorder affects mostly girls with an apparently normal prenatal and perinatal development who begin deteriorating at around five months of age and show a deceleration of head growth, loss of fine-motor hand skills with subsequent development of characteristic stereotyped hand-washing-like movements, a loss of social interactive abilities, poorly coordinated gait or trunk movements, and severe psychomotor and language retardation.

The provisional nature of the autism spectrum is highlighted by the lack of specific criteria for PDD-NOS, which is applied when severe and pervasive impairments are present in social interactions and verbal or nonverbal communication or there is a limit in the range of interests and behaviors but without meeting specific criteria for one of the other defined disorders.

Estimates of the prevalence of PDD have increased dramatically over the past several decades by about an order of magnitude, although the question of whether this represents a true increase in incidence versus increased recognition of a broader range of severity remains unsettled. Current estimates suggest that the overall prevalence for PDD is about 6 in 1000 [2, 3]. Awareness that the prevalence of PDD is markedly higher than previously thought has sparked understandable concern regarding an apparent epidemic of autism and related disorders. If increased prevalence does represent increased incidence, such an epidemic would be the result of environmental factors either working independently or interacting with vulnerability genes. We will not review the extensive relevant literature, much of it in the lay press or on the Internet, but we note overwhelming evidence that genetic factors account for over 90% of the phenotypic variance in autistic disorder [4]. Accordingly, several international collaborations are intensively seeking specific genes that convey vulnerability for PDD, beyond the *MECP2* gene, which is now known to cause Rett's disorder [4]. The presumption is that identification of specific genes and their associated neurochemical pathways will eventually provide the framework necessary to make advances in the diagnosis and therapeutics of PDD, but that time has not yet arrived. In the meantime, treatment is essentially empirical. There is not yet a cure for autistic disorder and until recently (see Risperidone section) medications had been approved by the U.S. Food and Drug Administration (FDA) for an indication related to autism or PDD. Thus, all pharmacotherapy in PDD is "off label," and our confidence in the current evidence base for clinical decisions varies from moderate to highly tenuous.

Current pharmacological strategies utilize drugs previously shown to be effective in other psychiatric disorders. Drugs are used to target specific symptom domains or comorbid disorders associated with the core triad of PDD. The intent is to use

medications to control interfering behavioral dysregulation so as to indirectly enhance the ability to benefit from educational services and behavior modification. In addition to the core symptoms of impaired social interactions and communication and restricted stereotyped interests, children and adolescents with PDD often present with, or later develop, interfering behaviors such as aggression, impulsivity, hyperactivity, inattention, or affective lability (temper tantrums), anxiety symptoms, and sleep disturbance [5–7]. An emerging, albeit provisional literature suggests an association between PDD and affective disorders, anxiety disorders, and Tourette's disorder [8–10]. Although, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), attention-deficit hyperactivity disorder (ADHD) cannot be diagnosed in the presence of PDD, a burgeoning literature indicates a high prevalence of hyperactivity and impulsivity symptoms in children and adolescents with PDD [6, 11]. The combination of autism and hyperactivity predicts markedly increased impairment and family distress, as indexed by higher rates of hospitalization and medication treatment [12].

In the absence of specific treatments, a wide range of medications have been used to treat symptoms associated with PDD. Recent surveys indicate that between 46 and 55% of individuals with autism take one or more psychoactive medications [13–15]. The most frequently prescribed drugs were antidepressants (22–32%), followed by antipsychotics and stimulants (17 and 14–20%, respectively), with fewer having been prescribed α_2 -agonist antihypertensive agents (clonidine, guanfacine), anxiolytics/hypnotics, mood stabilizers, and anticonvulsants. A majority also reported using complementary or alternative treatments, including vitamin supplements (43%) and special diets (27%) [13], though these treatments will not be discussed further in this chapter.

In this chapter, we will first review the evidence supporting the therapeutic use of the major classes of psychotropic agents. We will then briefly suggest approaches for the treatment of children and adults with PDD which are based on commonly encountered clinical syndromes.

12.2 PHARMACOLOGICAL STUDIES IN AUTISM SPECTRUM DISORDERS

12.2.1 Serotonergic Medications

Ever since whole-blood serotonin (5-hydroxytryptamine, or 5-HT) levels were found to be elevated compared to controls in about one-third of drug-free individuals with autism [16], such hyperserotonemia (25–50% above normal) has been consistently replicated [17]. Despite extensive work [18–20], the mechanisms responsible for increased whole-blood serotonin (WBS) levels, their relation to central serotonin metabolism and whether either correlates with PDD symptoms have yet been determined. Nevertheless, the resulting hyperserotonin hypothesis led to therapeutic trials of fenfluramine, an indirect serotonin agonist, with initial positive effects in a case series and a large single-blind study [21, 22], which were followed by disappointing reports of increased repetitive behaviors and global clinical worsening in double-blind, placebo-controlled trials [23–25]. The post hoc explanation that chronic fenfluramine administration likely results in compensatory downregulation of central serotonergic signaling in the central nervous system (CNS) was supported

by a similar finding of clinical worsening in a double-blind, placebo-controlled study of acute dietary tryptophan depletion [26].

The availability of serotonin reuptake inhibitors and their dramatic effects on decreasing the repetitive behaviors and intrusive thoughts of obsessive-compulsive disorder suggested that they might be similarly efficacious in PDD. These studies began with the first available, nonselective but potentially serotonergic agent, clomipramine.

12.2.1.1 Clomipramine. Clomipramine is a tricyclic antidepressant that strongly inhibits reuptake of serotonin but also has substantial noradrenergic, anticholinergic, and histaminergic effects. An encouraging initial case series [27] and an open-label study [28] reported improvement of social relatedness and reduction of obsessive-compulsive symptoms, aggression, and impulsivity in adolescents and adults treated with clomipramine. A double-blind crossover study of clomipramine versus desipramine and clomipramine versus placebo in two separate groups of adolescents with autism indicated that clomipramine was superior to placebo and desipramine on measures of anger and obsessive-compulsive symptoms and superior to placebo but not to desipramine for controlling hyperactivity [29]. Significant adverse effects with clomipramine included tachycardia, prolongation of the QT interval, and a grand mal seizure, each occurring in one subject. Increased irritability, temper outbursts, and aggression occurred with desipramine. In contrast with this earlier study's results, an open pilot study with younger children with autism (age range 3.5–8.7 years) found that only one of eight patients appeared to improve with clomipramine at doses ranging from 2.5 to 4.6 mg/kg/day. Additionally, adverse effects, including drowsiness, aggression, irritability, temper tantrums, increased self-injurious behavior, and insomnia, were common in this group of children. In another case series of five prepubertal boys with autistic disorder and severe mental retardation, doses in the range of 3–5 mg/kg/day produced improvement in all five, including marked improvement in three. However, dose elevation to 200 mg/day (3.9–9.8 mg/kg/day) produced extreme agitation, aggression, tremors, anorexia, and insomnia and ultimately required hospitalization in three [28]. Additionally, in a double-blind, placebo-controlled crossover comparison of clomipramine and haloperidol in children and adults with autistic disorder, clomipramine was less effective than haloperidol and caused a higher incidence of adverse effects. The exploration of clomipramine has ceased since that time owing to the high incidence of its broad range of adverse effects, especially in younger patients. The availability of newer selective serotonin reuptake inhibitors (SSRIs) with lower risks of cardiotoxicity and fewer autonomic effects resulted in a shift to studies of SSRIs for the treatment of PDD.

12.2.1.2 Fluvoxamine. Following case series on the benefits of fluvoxamine in controlling repetitive behaviors in young adults with autism [30, 31], McDougle et al. published a double-blind, placebo-controlled trial of fluvoxamine for the treatment of adults with autistic disorder [32]. Eight of the 15 subjects treated with fluvoxamine (25–250 mg/day) were classified as responders as opposed to none in the placebo-treated group. Outcome measures included significant improvements compared with placebo on the clinical global impressions (CGI) scale, Yale–Brown obsessive-compulsive scale (Y-BOCS), Vineland maladaptive behavior subscales,

Brown aggression scale, and Ritvo–Freeman real-life rating scale. Fluvoxamine significantly improved language use and reduced repetitive thoughts and behaviors, aggression, and maladaptive behaviors. Adverse effects were relatively benign with only mild to moderate sedation (two patients on active medication) and gastrointestinal discomfort (three patients in the treatment group experienced nausea, resolved after the first two weeks of treatment). In children and adolescents with PDD, the evidence of beneficial effects from treatment with fluvoxamine has been less convincing. Although marked improvement after fluvoxamine at doses of ~ 100 mg/day was reported in a single case [33], a double-blind, controlled study, reported only in summary form as unpublished data [34] or as personal communication [35], failed to find significant benefit from fluvoxamine in children with PDD (dose range 25–250 mg/day, mean dose 107 mg/day). While only 1 of the 18 children randomized to fluvoxamine showed significant improvement, adverse effects were found in 14 and included insomnia, agitation, motor impairment, hyperactivity, aggression, irritability, anxiety, anorexia, decreased concentration, and increased impulsivity and appetite. A recent open-label trial of “low-dose” fluvoxamine (1.5 mg/kg/day) in 18 children and adolescents with PDD found no significant effects on primary or secondary outcome measures. In categorical analyses, 3 patients (17%) were classified as full responders, 5 (28%) as partial responders, and 10 (55%) as nonresponders [35]. Adverse effects were reported in 13 (72%), although most were mild and transient. However, akathisia/agitation/behavioral activation was reported in half of the sample and was severe enough to require discontinuation in three patients. The authors were impressed that all four female patients appeared to improve and suggested that sex may predict treatment response. However, the authors insisted on cautious interpretation of their results because of the lack of blinding and small sample size and acknowledged that fluvoxamine cannot yet be recommended for the routine treatment of anxiety or repetitive symptoms in pediatric subjects with PDD [35].

12.2.1.3 Fluoxetine. Small case series and larger open studies (totaling about 200 subjects) in adults, adolescents, and children with PDD have been published documenting improvements after fluoxetine for some patients, with regard to symptoms of irritability, withdrawal, temperament, social interaction, and repetitive behaviors [36–41]. In general, doses were in the range of ~ 20 mg/day, but some were as high as 80 mg, and several patients were reported to worsen on such higher doses. Fluoxetine was well tolerated in most, but adverse effects reported in some included transient appetite suppression, increased hyperactivity, irritability, and agitation. More serious adverse effects of fluoxetine have also been reported in patients with PDD [42]. In one case series, three children with Asperger’s disorder (ages 9–10 years old) developed hypomania on fluoxetine 20 mg/day which resolved with divalproex treatment [43]. In another case series, four boys, ages 6–10 years, developed extrapyramidal side effects with fluoxetine ($n=3$, dose 1–2 mg/day) or paroxetine ($n=1$, age 6 years, dose 10 mg/day) within days of starting treatment [44].

An extensive open study followed 129 children with PDD, ages 2–8 years, for an average of about 3 years (range 5–76 months) treated with fluoxetine [37]. The authors reported that 69% of young children with PDD had a favorable response to fluoxetine, which was typically sustained. The major predictor of beneficial response appeared to be family history of major affective disorder. Of those with a positive

family history of affective disorder, 78% were characterized as having an excellent or good response, versus 39% of those without such a family history [37].

Recently, Hollander et al. published the first placebo-controlled crossover study of fluoxetine in children and adolescents with PDD [45]. Fluoxetine and placebo were administered for eight weeks each with an intervening four-week washout period and mean doses of 9.9 ± 4.4 mg/day. Ratings on the children's Y-BOCS (C-YBOCS), used as a measure of repetitive behaviors, improved significantly, but ratings of global improvement in autistic symptoms did not. The authors ascribed this to order effects in a crossover design. Adverse effects of activation, sedation, diarrhea, and anorexia were seen in some of the patients but were generally in the mild range. Further, there was no statistically significant difference between those who required dose reductions on placebo as opposed to fluoxetine.

12.2.1.4 Sertraline, Citalopram, Escitalopram, and Paroxetine. To date, no placebo-controlled studies have been carried out to examine the efficacy of SSRIs other than fluoxetine and fluvoxamine for patients with PDD. The effects of sertraline have been described in a series of case reports and open studies of small samples of children and adults with PDD. A small open-label study of sertraline was conducted in 9 adults with mental retardation (age range 20–47 years old, 6 males), including 5 with comorbid autistic disorder. All had been admitted to an inpatient unit for self-injurious behavior and aggression [46]. Eight of 9 improved; only 1 patient needed to discontinue sertraline due to worsened self-picking and agitation. Dosage ranged from 25 to 150 mg/day. Unfortunately, concomitant psychotropic medications, including antipsychotics, stimulants, anticonvulsants, lithium, buspirone, and propranolol, were being prescribed for these patients, thus complicating the ability to ascribe efficacy to any specific agent. In two small case series in children with PDD (totaling 11 patients, age range 6–13 years), sertraline was described as effective in decreasing anxiety and compulsive behaviors at generally low doses, ranging from 25 to 50 mg [47, 48]. Adverse effects of behavioral activation and stomachaches were noted in a minority of patients [49].

The use of citalopram for PDD was reported in a retrospective chart review of 15 children and adolescents (13 males, age range 6–16 years, dose range 5–40 mg/day, treated from 14 to 624 days) [50]. All but 1 of the patients had prior trials of psychotropic medication, and 9 of the responders in this study had failed to improve with other SSRIs. The authors noted improvement on symptoms of anxiety, mood, preoccupations, and stereotypies for some patients. Reported adverse effects were mild and included headaches, sedation, increased aggression, agitation, and “lip dyskinesias.”

The pure levo isomer, escitalopram, was examined in a prospective 10-week open-label trial in children and adolescents with PDD ($n = 28$, age range 6–17 years old, 25 males, doses range 2.5–20 mg/day). Seventeen of the 28 improved significantly with escitalopram. The most prominent outcome was a significant decrease in irritability. One-quarter of the patients responded to low doses below 10 mg and subsequently did not tolerate doses above 10 mg, whereas another third of the patients only responded at a dose of 10 mg or higher. For the subjects that completed the study, dose-related adverse effects included irritability and hyperactivity, but there was no increase noted in either sleep disturbance or self-injurious behavior.

In the only report with paroxetine, irritability, temper tantrums, and interfering preoccupations were decreased in one seven-year-old boy with autism treated openly.

In order to approve an indication for a medication, the FDA requires that at least two positive trials that meet specific quality criteria, including blinding, adequate sample size, and appropriate statistical controls. This requirement was instituted to protect against type I error, which is expected to produce false-positive results at least 5% of the time. None of the SSRIs considered for PDD would currently meet the FDA standards. Thus, treatment of PDD with SSRIs must still be classified as empirical. However, if SSRIs are considered as a class rather than as individual agents, then modest claims can be asserted supporting efficacy for the treatment of repetitive behaviors, particularly as indexed by Y-BOCS ratings. Improvement in other associated symptoms, including global ratings of PDD psychopathology, was not replicated in the two controlled studies completed to date [32, 45].

12.2.2 Dopaminergic Medications

Neurochemical studies focusing on dopamine have been inconclusive. Measurements of urinary excretion of dopamine and its catabolites (homovanillic acid and 3,4-dihydroxyphenylacetic acid) have shown increased levels in some small studies but failed to show differences in a larger study [51]. Five out of seven studies of cerebrospinal homovanillic acid found no differences between controls and patients with autism, although two studies reported elevated levels in 50% of the patients [17]. Thus a possible role for dopamine in the pathophysiology of PDD is only supported indirectly by the results of drug treatment studies.

12.2.2.1 Typical Antipsychotics. Conventional antipsychotic drugs have frequently been prescribed for patients with PDD. Within this group of drugs, haloperidol has been the most intensively studied.

12.2.2.1.1 Haloperidol. Haloperidol has been shown to be useful in decreasing motor stereotypies, hyperactivity, and temper tantrums in both short- and long-term double-blind, placebo-controlled studies in children with autistic disorder [52–56]. However, adverse reactions such as sedation, acute dystonia, dyskinesias, and cognitive blunting have also been reported. In a double-blind, controlled longitudinal study, Campbell and colleagues [57] systematically monitored haloperidol-related dyskinesias in 118 children (95 boys, ages 2.3–8.2 years). The mean duration of the treatment was 708 days (range 25–3610), and the mean dosage was 1.7 mg/day. Dyskinesia developed in 40 subjects (34%), 12% developed tardive dyskinesia, and 80% presented withdrawal dyskinesias. Multiple dyskinetic episodes were more common in the subgroup of children with the longest durations of treatment, which was related to higher cumulative dose but not higher daily dose [57].

12.2.2.1.2 Pimozide. The only other typical neuroleptic agent compared in a control study for autism is pimozide. In a multicenter double-blind crossover study of 34 children with autistic disorder, pimozide (1–9 mg/day) was found to be as effective as haloperidol in controlling aggression toward others but not in controlling self-injury [58].

Unlike the usual situation with most psychotropic medications, the literature on the efficacy and safety of typical antipsychotics for PDD is limited to studies of children and adolescents. Thus, overall, the literature indicates that typical antipsychotics are efficacious for ameliorating symptoms of aggression, affective lability, and withdrawal associated in children and adolescents with PDD. However, the substantial risks of extrapyramidal side effects, along with affective and cognitive blunting, particularly at higher doses, have made physicians reluctant to prescribe typical neuroleptics as first-line medications in PDD.

12.2.2.2 Atypical Antipsychotics. Over the past 15 years, considerable interest has been generated by the introduction of the “atypical,” or second-generation, antipsychotic agents, because of their potentially safer profile with regard to the risk of extrapyramidal symptoms and their greater efficacy on psychotic negative symptoms in schizophrenia, such as social withdrawal, blunted affect, difficulty in abstract thinking, and lack of spontaneity [59]. These presumed advantages have encouraged clinicians to prescribe atypical neuroleptics for other indications besides psychotic disorders, including the manifestations of PDD.

12.2.2.2.1 Clozapine. Clozapine is the prototypical medication in this class, and as with the other medications discussed in this section, its pharmacological properties and receptor profiles are discussed extensively elsewhere in this book [60, 61]. Two small reports described the use of clozapine in PDD. In one study, two of three children with marked hyperactivity, fidgetiness, and aggression, who had been nonresponsive to prior typical antipsychotic treatment, improved for up to 8 months with clozapine at 100 mg/day. In the third participant, after an initial improvement, measures of symptom severity returned to baseline after 5 months, despite doses of up to 450 mg/day [61]. In a single case report, a 15-day trial of clozapine reduced severe aggression which had not responded to previous treatments with multiple psychotropics, including typical and atypical antipsychotics [60]. The high risks of agranulocytosis, new-onset seizures, severe weight gain, severe sedation, and drooling all limit the use of clozapine. Concerns about clozapine are heightened in PDD because of the higher incidence of seizures and the requirement to check white blood cell counts with regular blood draws, which are particularly challenging in many children with PDD. For all these reasons, in the face of a lack of controlled studies addressing safety and efficacy, clozapine remains the last resort, when all other potential medications, administered for adequate durations and at adequate doses, have demonstrated no beneficial effect.

12.2.2.2.2 Risperidone. Multiple open-label studies with risperidone [62–68] and several double-blind, placebo-controlled trials have confirmed the clinical efficacy of risperidone in adults, adolescents, and children with PDD [69–72]. The largest was a multisite, randomized, double-blind, placebo-controlled study conducted by the National Institute of Mental Health (NIMH) Research Units on Pediatric Psychopharmacology (RUPP) Autism Network [69, 71]. The primary aims of the RUPP study were to evaluate the short-term efficacy and safety of risperidone in children and adolescents with PDD and to determine whether observed clinical benefits persisted. The study was divided into three phases: Phase 1 was an eight-week, double-blind, randomized, parallel-group comparison of risperidone and placebo.

Subjects assigned to placebo who did not improve were offered an eight-week open label trial with risperidone. Phase 2 consisted of open-label treatment with risperidone for 4 months for patients who had improved in the eight-week acute trial. Phase 3 was a randomized, double-blind, placebo-controlled discontinuation study.

After eight weeks of phase 1, risperidone at doses between 0.8 and 3.5 mg/day reduced irritability, tantrums, aggression, and self-injurious behaviors as measured by the autism behavior checklist in 57% of the sample versus 14% receiving placebo, with a reported improvement rate of 69% with risperidone versus 12% with placebo. Among the children with a positive response to risperidone at eight weeks, two-thirds continued to benefit from the treatment after six months [71]. In a subsequent report on phase 2 (the four-month open-label study), the average autism behavior checklist irritability score showed a statistically significant increase over time, but the absolute change was small and not clinically significant, particularly when compared with the pretreatment score. Of the 32 children who completed the third phase of the study (randomized, double-blind, placebo-controlled discontinuation phase), randomization to gradual placebo substitution led to a high rate of relapse (63%) compared to those randomized to continue risperidone (12%) [71]. In October 2006 the U.S. Food and Drug Administration (FDA) approved risperidone for the symptomatic treatment of irritability in children and adolescents (ages 5–16 years) with autism. The approval was the first for the use of a drug to treat behavior associated with autism in children. The targeted behaviors include aggression, deliberate self-injury, and temper tantrums.

Overall, risperidone has been shown to be efficacious in controlling maladaptive behaviors (e.g., aggression, irritability, self-injury, hyperactivity) in adults and children with PDD, improvements are stable over several months, and discontinuation in responders causes relapse. Furthermore, a secondary analysis of the RUPP study showed that while risperidone did not improve the social communication impairments typical of PDD, it did significantly improve repetitive interests and behaviors [73]. Thus, improvements in the social domain reported in some small open-label studies [63, 68] might be indirectly related to control of maladaptive behaviors rather than to direct actions of the medication. Although small open-label studies have reported risperidone to be beneficial and safe over longer period of time (from six months to three years), its long-term efficacy and safety remain unknown.

12.2.2.2.3 Olanzapine, Quetiapine, Ziprasidone, and Aripiprazole. Several small, open-label studies have suggested that other atypical antipsychotic such as olanzapine [74–76] may be beneficial in reducing irritability, impulsivity, hyperactivity, self-injury, aggression, and interfering repetitive behaviors in autistic children and adolescents. In a small, randomized, parallel-group comparison of haloperidol and olanzapine (six children per group, mean age 8 ± 2 years), Malone and colleagues found equivalent symptom reduction and adverse effects with both drugs, although weight gain was significantly greater on olanzapine [75].

In an open label trial of quetiapine in six males with PDD (mean age 10.9 ± 3.3 years) [77] and in another trial with nine males with PDD (mean age 14.6 ± 2.3 years) [78], only two in each study were classified as responders, leading to the conclusion that quetiapine may not be particularly effective in the treatment of adolescents with autism [78]. Those prospective findings were in conflict with a retrospective study

which suggested that quetiapine might be modestly effective in children and adolescents with PDD [79].

In individuals with autism, ziprasidone appeared to have a beneficial effect on maladaptive behaviors in a case series of children, adolescents, and young adults with autistic disorder [80]. Switching to ziprasidone from other atypical antipsychotic agents, with the goal of improving major health indices including weight and lipid levels, has been reported beneficial in an open-label trial studied retrospectively in adults with autistic disorder [81].

Aripiprazole is a new compound with a distinct profile from the other atypicals, as it is a partial D_2 agonist, 5-HT_{1A} agonist, and 5-HT_{2A} antagonist [82]. A single case series reported on the tolerability of aripiprazole in five children with PDD (mean age 12 years, age range 5–18 years) at a mean dose of 12 mg/day for at least eight weeks. During this short-term trial, aripiprazole was beneficial and well tolerated with no significant side effects on heart rate or blood pressure. Weight loss was reported (mean loss 3.7 kg), and was mostly attributed to the subject's discontinuation of previously used atypical neuroleptics before beginning the aripiprazole trial [83].

The most frequent adverse effect reported with atypical antipsychotics is weight gain, particularly in children and adolescents. In the risperidone RUPP study, average weight gain on risperidone was 2.7 kg versus 0.8 kg on placebo. Weight gain is associated with all atypicals except possibly ziprasidone and aripiprazole [69]. Treatment of children with PDD tends to be chronic. Accordingly, the consequences of weight gain must not be overlooked, as they can lead to complications such as hyperglycemia, diabetes, and hepatotoxicity [84, 85]. Increased prolactin levels have also been reported following treatment with atypical neuroleptics, although the pathophysiological implications are not known, especially in the absence of signs or symptoms such as galactorrhea, gynecomastia, or amenorrhea [86]. In young children, tachycardia and prolongation of the QT interval have been reported [87], and a sudden cardiac-related death was reported in a 34-year old woman treated with risperidone [88]. Additionally, despite early expectations that the atypicals would have decreased risks of serious extrapyramidal adverse effects such as tardive dyskinesia, there have been case reports of tardive dyskinesia following treatment with atypical agents, demonstrating, at the very least, that these newer agents are not free of such risks. Other adverse effects of risperidone in PDD include increased appetite, fatigue, drowsiness, dizziness, and hypersalivation [69].

In summary, despite the reassurances provided by small open-label studies of risperidone that reported maintenance of benefits and reasonable safety over periods of six months to three years, the important question of safety for long-term use of risperidone, as well as other atypical antipsychotics, remains unsettled [68, 89, 90]. Increasingly, concerns regarding metabolic effects on weight, glucose tolerance, and lipid levels need to be addressed in long-term studies that will incorporate novel developments in pharmacogenomics. The metabolically more benign alternatives, aripiprazole and ziprasidone, have yet to be tested in randomized controlled trials, but such studies are clearly needed.

12.2.2.3 Stimulants. Despite the lack of inclusion in the DSM-IV, the prevalence of impairing symptoms of hyperactivity, impulsivity, and inattention in children with PDD is estimated to range from 13 to 59% [11, 91]. Hyperactivity/impulsivity and/or inattention, when combined with PDD, are associated with marked increases in

impairment and family distress, resulting in higher rates of hospitalization and treatment with medications [12]. The established efficacy of methylphenidate in children with ADHD [92, 93], along with rising expectations for academic productivity of children with PDD, has led to a substantially increased rate of prescribing stimulants for children and adolescents with PDD combined with hyperactivity, impulsivity, and/or inattention. In the 1970s, Campbell and colleagues reported that amphetamine treatment for children with PDD was associated with an increased rate of stereotypies and irritability [94, 95]. Subsequent open-label studies also reported exacerbations of stereotypies, irritability, tics, and aggression, thereby discouraging the use of stimulants in PDD [96].

12.2.2.3.1 Methylphenidate. Twelve studies have been published on the use of methylphenidate in PDD. Two of these were small placebo-controlled, double-blind studies of 10 [97] and 13 children and adolescents [98], and one is a recently completed multisite trial [99]. The remaining studies have been case reports [100–105], two open trials [106, 107], and a randomized trial in a single child [108]. The clinical literature suggests that a sizable proportion of children with autism may be more susceptible to the adverse effects of these medicines. IQ below 48 [109] has been supported as a robust variable moderating clinical response and tolerability [110], that is, individuals with lower IQ scores tend to improve less and have more frequent adverse effects.

In a retrospective analysis of 195 children with PDD (174 males; age 7.3 ± 3.4 years), 25, 23, and 11% of patients with a history of one, two, or three stimulant trials, respectively, responded positively to their first stimulant trial [111]. Patients with Asperger's disorder, in contrast to those with autistic disorder or PDD-NOS, were significantly more likely to respond to a stimulant trial. No association was found between stimulant type or IQ and response. However, medication response was positively affected in those 18.2% trials where concomitant psychoactive medications were prescribed. Adverse effects, including agitation, dysphoria, and irritability, frequently occurred [111]. The investigators concluded that stimulants appeared to be largely ineffective and poorly tolerated for the majority of patients with PDD, although the response may differ with PDD subtype.

To investigate the efficacy and safety of the most frequently prescribed stimulant, methylphenidate, in a large sample and study the effects of age, IQ, diagnosis, and dosage of methylphenidate as response moderators, the RUPP group recently completed a double-blind, placebo-controlled, crossover trial followed by an open-label continuation phase with methylphenidate [99, 110]. Seventy-two drug-free children (5–14 years of age) with PDD and severe to moderate hyperactivity first underwent a test dose phase to establish tolerability. A total of 66 tolerated methylphenidate and were randomized to a four-week crossover placebo-controlled blinded treatment with methylphenidate at three doses (low, medium, and high were 0.1, 0.125, and 0.5 mg/kg/day, respectively). Methylphenidate significantly decreased both parent and clinician measures of hyperactivity with no dose-related differences. Effect sizes ranged between 0.2 and 0.5, which is substantially smaller than those obtained for methylphenidate in children with ADHD [112]. The high-dose condition was associated with significantly poorer outcomes on measures of "lethargy/social withdrawal." Neither, age IQ, diagnosis, or body weight moderated treatment responses. However, in accordance with the observations reported in earlier studies, children with Asperger's disorder or with PDD-NOS were more likely to be classified

as responders. Overall, 18% of subjects discontinued the trial because of adverse effects (6 during the test dose phase and 7 during the double-blind crossover phase). The most frequent significantly increased adverse effects were decreased appetite at medium and high doses, difficulty falling asleep at all doses, and irritability and emotional outbursts at medium doses [99, 110].

Overall, current evidence suggests that treatment with methylphenidate in PDD, when effective, is exclusively symptomatic and affects hyperactivity and impulsivity primarily, along with other related disruptive behaviors (e.g., temper tantrums, oppositional behaviors). The administration of a single test dose of methylphenidate in the clinician's office can be used to minimize the risks of a more prolonged adverse response and to improve the likelihood of identifying PDD subjects who can benefit from ongoing methylphenidate treatment [107].

12.2.3 Miscellaneous Agents

12.2.3.1 α_2 -Adrenergic Agonists. Biochemical studies of the noradrenergic system have shown little evidence of specific abnormalities related to PDD. Differences have not been found in noradrenergic metabolites in urine or cerebrospinal fluid; increased levels in several studies of peripheral blood have been reported but might be related to the increased arousal associated with the stress of venipuncture [17]. Because α_2 -adrenergic agonists have been shown to be beneficial in controlling hyperactivity and inattention in other developmental disorders, they have also been considered for targeting these symptoms in patients with PDD [113].

12.2.3.1.1 Clonidine. Clonidine is an α_2 -adrenergic receptor agonist that has been shown to be potentially efficacious in reducing impulsivity, inattention, and hyperactivity combined with PDD. In a four-week double-blind, placebo-controlled crossover study with transdermal clonidine in nine individuals with autistic disorder (ages 5–33 years), subjects received either clonidine (nominally 0.1–0.3 mg/day) or placebo by a weekly transdermal patch. Clonidine treatment was associated with significant improvement in measures of social relationship, affective responses, sensory responses, hyperactivity, and anxiety. Adverse effects included sedation and fatigue, which were most prominent during the first two weeks of clonidine treatment.

Another small placebo-controlled, double-blind crossover trial of clonidine in eight children (ages 5–13 years) with autistic disorder and symptoms of inattention, impulsivity, and hyperactivity reported improved irritability, stereotypies, hyperactivity, and inappropriate speech, as measured by parents and teachers. However, when clinicians rated videotaped sessions of these children at the beginning and end of each crossover arm, there was no significant difference between clonidine and placebo conditions. The authors noted that the videotaped sessions were highly structured and were in a physically small space and as a result may not truly have been representative of the children's every-day experiences [114]. Of note, in following up the individual children, only two of the children continued on clonidine for more than one year, while four others, who had initially shown improvement in the acute treatment phase, became tolerant to the effects and then could not tolerate dose increases due to hypotension, irritability, or sedation. Thus, the evidence

supporting potential efficacy of clonidine in PDD is uncertain, particularly given the small samples studied in these pilot trials.

12.2.3.1.2 Guanfacine. There are no controlled studies on the efficacy and safety of guanfacine in individuals with PDD. In a retrospective chart review of 80 subjects, Posey et al. reported that guanfacine (mean dose 2.5 ± 1.7 mg/day) was beneficial in controlling hyperactivity, inattention, tics, and insomnia in 24% of the patient. PDD-NOS and Asperger's disorder diagnosis as well as absence of mental retardation predicted a greater rate of positive response [115].

12.2.3.2 Medications with Mixed Monoaminergic Mechanisms: Mirtazapine and Venlafaxine. Although mirtazapine and venlafaxine have different mechanisms of action, they are both antidepressants primarily acting on both serotonergic and noradrenergic systems, without the substantial histaminergic, cholinergic, and α -adrenergic effects of the tricyclic drugs. Mirtazapine blocks the noradrenergic autoreceptor as well as the 5-HT₂ and 5-HT₃ serotonin receptors; venlafaxine potentially inhibits the reuptake of serotonin and norepinephrine.

12.2.3.2.1 Mirtazapine. Posey et al. described a naturalistic, open-label study of mirtazapine in children and young adults with PDD ($n = 26$, 21 males, age range 3.8–23.5 years, mean age 10 years, followed for an average of five months) [116]. All but 1 of the 26 subjects had undergone previous trials of psychotropic medication, averaging 5.5 prior trials per individual. In the study, patients received doses ranging from 7.5 to 45 mg/day; 17 continued concurrent psychotropic medication, including anticonvulsants, stimulants, atypical neuroleptics, and SSRIs. Outcome measures included the CGI and aberrant behavior checklist as well as an adverse effects checklist. Of the group, 25 of 26 remained in treatment for at least four weeks. Nine were classified as “much improved” or “very much improved.” Mean aberrant behavior checklist scores at baseline and after treatment did not differ significantly. Patients classified as improved had decreased levels of a variety of symptoms, including aggression, self-injury, irritability, hyperactivity, anxiety, depression, and insomnia, but the core symptoms related to social behavior and communication were unaffected by mirtazapine. Adverse effects were reported as mild, and included increased appetite, sedation, and irritability. Responders and nonresponders could not be differentiated with respect to age or final mirtazapine dose.

12.2.3.2.2 Venlafaxine. A single open-label, retrospective study in children and adolescents with PDD has addressed the potential utility of venlafaxine ($n = 10$, age range 3–21 years). Primary diagnoses included 4 subjects meeting criteria for autistic disorder, 5 for Asperger's, and 1 with PDD-NOS. Comorbid diagnoses included symptoms of ADHD, body dysmorphic disorder, separation anxiety disorder, obsessive-compulsive disorder, and Tourette's disorder. Six of 10 subjects had CGI ratings of “very much improved” on venlafaxine [117]. Improved symptoms in responders included symptoms of communication, language use, social deficits, obsessional/repetitive symptoms, and symptoms related to hyperactivity, impulsivity, and inattention.

12.2.3.3 Glutamatergic Medications. Several investigators have recently explored the potential usefulness of compounds that modulate glutamate neurotransmission. As there is no direct evidence of glutamate alteration in individuals with PDD, consideration of potentially therapeutic utility in PDD is based on a theoretical analysis of the role of glutamate in brain development and potentially in autism [118, 119]. Each study includes its own rationale, none of their hypotheses can be characterized as compelling, and results are highly preliminary.

12.2.3.3.1 Amantadine. Amantadine is a noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist. It is used to treat herpes zoster, influenza, and Parkinson's disease. A single double-blind, placebo-controlled trial on the efficacy and safety of amantadine enrolled 39 children and adolescents with autistic disorder [119]. After a placebo lead-in phase to assess patient compliance with a double-blind protocol, subjects were randomly assigned to receive either amantadine or placebo for a four-week treatment period. Dosages were 2.5 mg/kg/day for one week followed by 5 mg/kg/day. While clinical ratings on the aberrant behavior checklist (ABC) hyperactivity factor improved significantly, with a positive response rate based on the CGI of 53% with amantadine compared to 25% with placebo, there were no significant differences on measures obtained based on parent ratings. Four subjects reported insomnia on amantadine versus 2 on placebo.

12.2.3.3.2 Lamotrigine. Lamotrigine is thought to act by inhibiting glutamate release through blockade of voltage-sensitive sodium channels and stabilization of the neuronal membrane [120]. It is effective in the treatment of focal epilepsies and has been shown effective in the treatment of bipolar disorder [121, 122]. In a double-blind, placebo-controlled parallel group study of 28 children with autistic disorder, lamotrigine was titrated to a mean dose of 5 mg/kg/day and maintained over four weeks. No significant effects were measured on the PDD core symptoms measured by the childhood autism rating scale, the autism behavior checklist, and the ABC scores of associated symptoms or measures of global functioning such as the Vineland adaptive behavior scale. Parent measures improved significantly for both medication and placebo groups, presumably reflecting expectation effects [123].

12.2.3.3.3 *d*-Cycloserine. The compound *D*-cycloserine is a noncompetitive partial agonist of glutamatergic NMDA receptors. At low doses, *D*-cycloserine has been shown to ameliorate negative symptoms of schizophrenia [124]. In a recent pilot study, three different doses of *D*-cycloserine were administered during three two-week periods to 11 drug-free subjects (age 5 years and older) with autistic disorder following a two-week, single-blind, placebo lead-in phase. Ratings included the CGI and aberrant behavior checklist. Ten subjects (eight males) completed the eight-week study. Significant improvements were found on the CGI and social withdrawal subscale of the ABC, and *D*-cycloserine was well tolerated at most of the doses used. Although very preliminary, these results suggest that *D*-cycloserine might be effective for specific aspects of the social impairment in autism [125]. Controlled trials appear to be warranted.

12.2.3.4 Antiepileptic Drugs. In addition to their proven seizure control activities, antiepileptic drugs (AEDs) have been used in children with PDD for the purpose of

controlling behavioral and mood disturbances. With the exception of the one controlled study with lamotrigine described above, a total of 12 case reports or open-label studies on AEDs in individuals with PDD have been published: 7 with valproic acid [126–132], 3 with carbamazepine [56, 133, 134], 1 open-label study with lamotrigine [135], and 1 with topiramate [136]. In total, these studies included 50 individuals between the ages of 22 months and 40 years. Affective symptoms (manic, hypomanic or depressive episodes) were recognized in 11 of these 50 individuals [129, 134, 137, 138], epilepsy in 20, and EEG abnormalities in the absence of clinical seizures in 8. Of the individuals with affective disorders, only 1 also had epilepsy and 1 other individual had an EEG with epileptiform activity [134]. In the cases in which AEDs were used to control seizures or epileptiform abnormalities, improvements in expressive and receptive language were also reported, although specific language assessment measures were not used in any of these cases [139]. Affective symptoms improved in 9 of the 11 patients with recognized mood disorders.

The diagnosis of affective disorders in children and adolescents with PDD is particularly challenging, partly owing to the impaired communication skills characteristic of this population. Additionally, in the course of a mood disorder episode, children with PDD can also present with changes in their communication skills. A positive response to an AED, in terms of communication skills, could be secondary to an improvement of affective symptoms. In the studies reviewed, about 85% of the patients treated with an AED showed diminished behavioral disturbances (as evidenced by decreased irritability, aggression, or maladaptive behaviors), regardless of whether or not seizure activity was controlled. In seven of nine studies with valproate, improvements in social skills were also reported [129, 139]. Despite hypothesized similarities in the way some AEDs may affect their pathophysiological mechanisms, the relationships between epilepsy, language development, and behavioral disturbances in PDD are not well understood. The evidence to date on the beneficial effect of AEDs in PDD (with or without epilepsy) is based only on case reports; placebo-controlled, double-blind studies are clearly needed.

12.3 TARGETING SYNDROMES WITHIN AUTISM SPECTRUM DISORDERS: PRAGMATIC CONSIDERATIONS

As discussed above, PDD and autistic disorder in particular are neurodevelopmental disorders involving social cognition throughout life, beginning with the earliest stages of postnatal development. Brain regions involved in social cognition (e.g., the amygdala, hippocampus, prefrontal cortex and other specific parietotemporal regions) are also implicated in other neuropsychiatric disorders such as major depressive disorder, schizophrenia, anxiety, obsessive-compulsive, and attention-deficit hyperactivity disorders. As no specific pharmacological treatments have been developed for autistic disorder or the other PDDs, current pharmacological strategies are limited to drugs previously shown to be effective in comorbid disorders or on symptoms often associated with the core triad of PDD. Four major groups of such symptoms should be considered: (1) motor hyperactivity and inattention, (2) anxiety and depression, (3) interfering repetitive phenomena, and (4) aggression toward others/self-injurious behaviors.

Motor hyperactivity and inattention are more common in prepubertal children than in adolescents or adults. Methylphenidate should be considered with an initial test dose of 0.4 mg/kg in the clinician's office [107], keeping in mind that only a few studies support their efficacy in PDD. Preliminary, albeit inconclusive data suggest that methylphenidate may be more effective in children with Asperger's disorder or PDD-NOS than in children with strictly defined autistic disorder [107, 111]. If a stimulant is not tolerated or is not beneficial, clonidine should be considered, although with careful monitoring for tolerability. When hyperactivity is associated with symptoms such as mood lability and aggression, risperidone may be considered the first choice before methylphenidate, particularly if metabolic adverse effects are less worrisome.

Mirtazapine or low doses of an SSRI may be useful in ameliorating **anxiety** or **depression** in patients with PDD. A positive family history of mood disorder may predict such efficacy [37]. Many clinicians believe that the potential for behavioral activation with SSRIs is inversely related to age, although no controlled study has examined this question systematically. Behavioral activation by an SSRI has been suggested as one of the possible factors associated with the observed increase in suicidal behaviors and ideation detected in combined analyses of SSRI-controlled trials conducted in pediatric subjects (see <http://www.fda.gov/cder/drug/antidepressants/>), leading to increased caution and concerns regarding the use of SSRIs in childhood and adolescence.

If behavioral therapy is ineffective or induces only partial improvement on **interfering repetitive behaviors**, low doses of an SSRI may be considered. Combining an SSRI with an atypical antipsychotic or a mood stabilizer may also be useful, both as an augmentation strategy or in order to prevent behavioral activation. Recent large controlled studies indicate that atypical antipsychotics, risperidone in particular, significantly improve not only severe tantrums, aggression, and self-injurious behaviors [71], but also restricted repetitive and stereotyped patterns of behavior [73].

Aggression and **self-injurious behaviors** can be among the most troublesome behavioral symptoms as they have a profound impact on suitability for appropriate educational and residential placements. In placebo-controlled studies, these behaviors tend to occur at very low base rates, because of the ethical constraints related to the potential assignment to placebo. Antipsychotics may be beneficial in some cases, although there is a stronger argument for the efficacy of careful functional behavioral analyses that detect the antecedents and the consequences that maintain maladaptive behaviors [140].

In all cases, the absence of clear evidence from randomized controlled trials requires that clinicians carefully identify target symptoms, establish pretreatment baselines, systematically evaluate progress and the potential emergence of adverse effects, and proceed step by step as much as possible. Although "rational polypharmacy" is no longer always an oxymoron and indeed has been relabeled "combined pharmacotherapy," the simultaneous use of several psychotropic agents inevitably complicates clinical decision making and treatment adherence and tends to lead to a ratchet-like increase in the complexity of the treatment plan. If psychotropic medications administered in PDD appear to be beneficial with acceptable adverse effects, they are likely to be used for considerable periods, thus justifying slow, deliberate periods of titration and judicious single-subject experimentation. With increasing frequency, clinicians are being confronted with children who may already

be taking multiple medications with equivocal or no evidence of improvement. In such situations, the mere suggestion of adverse effects should be a sufficient basis to advocate a tapered washout, ideally, one by one, to reestablish a baseline from which future decisions can be made in collaboration with the child's caregivers. Medications in PDD are, at best, supportive, and their utility also needs to be regularly examined in terms of the child's overall context. Thus, some children may need pharmacological support to engage optimally in some educational and habilitative settings but not in others. The latter environment is a priori preferable but is not always available.

12.4 CONCLUSIONS

As this review has documented, there are no currently available medications that improve deficits in social interaction and communication, which are among the core symptoms of PDD. Still, fragmentary but accumulating evidence suggests that reducing interfering repetitive, stereotyped, hyperactive/impulsive, and aggressive behaviors through the judicious administration of psychotropics may allow some children with PDD to more fully access the educational and psychosocial interventions that may be crucial for future improvements in communicative, social, and academic skills. Larger controlled comparison trials are urgently needed to optimize the current pharmacological armamentarium while waiting for new insights from ongoing molecular genetic and neuroscience studies to yield novel treatments not yet imagined.

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13

STROKE: MECHANISMS OF EXCITOTOXICITY AND APPROACHES FOR THERAPY

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13.1	Stroke	348
13.1.1	Introduction and Symptoms	348
13.1.2	Prevalence and Incidence	349
13.1.3	Other Types of Stroke and Stroke-Related Brain Injury	350
13.2	Mechanisms of ischemic brain injury	351
13.3	Animal models of global and focal ischemia	353
13.4	Glutamate receptors and development of NMDA receptor antagonists	354
13.4.1	Glutamate and Glutamate Receptors	354
13.4.2	MK-801 : The “Gold Standard”	355
13.4.3	Other NMDA Antagonists	356
13.4.3.1	Studies with Other Competitive and Noncompetitive NMDA Antagonists	356
13.4.3.2	Polyamine Site Antagonists of NMDA Receptor	357
13.4.3.3	Glycine Site Antagonists of NMDA Receptor	357
13.4.4	Clinical Data	358
13.5	Summary of issues with preclinical stroke studies using NMDA antagonists	359
13.5.1	Side Effects and Other Issues with Development of NMDA Antagonists	359
13.5.2	Time Window and Other General Issues	360
13.6	Other glutamatergic approaches	361
13.6.1	AMPA Receptor Antagonists	361
13.6.1.1	Studies with NBQX and Related Quinoxalinediones	361
13.6.1.2	GYKI 52466 and Related 2,3-Benzodiazepines	362
13.6.1.3	Decahydroisoquinolines	362
13.6.1.4	Summary of Issues with Preclinical Stroke Studies Using AMPA Antagonists	362
13.6.2	Kainate Receptor Antagonists	363
13.6.3	Metabotropic Glutamate Receptors (mGluRs)	363
13.6.4	Glutamate Transporters	365

13.7	Upstream and downstream neuroprotective approaches	366
13.7.1	Glutamate Release Inhibitors: Calcium and Sodium Channel Blockers (Up-stream Approaches)	366
13.7.1.1	Neuronal Calcium Channel Blockers	366
13.7.1.2	Sodium Channel Blockers	367
13.7.2	Anti-Inflammatory Agents, Antioxidants, Caspase and Other Antia-poptotic Approaches (Down-Stream Approaches)	367
13.7.2.1	Inflammatory Pathways	368
13.7.2.2	Cytokine Inhibition	368
13.7.2.3	Chemokine Inhibition	369
13.7.2.4	p38 Inhibition	369
13.7.2.5	Antiadhesion Molecules	369
13.7.2.6	Nitric Oxide Synthase Inhibition (Both Anti-inflammatory and Antioxidant Action)	370
13.7.3	Antioxidants	371
13.7.4	Apoptosis and Caspase Inhibitors	372
13.8	Some suggested criteria for development of neuroprotective drug	374
13.9	Growth factors, functional recovery, and approaches to repair brain poststroke	375
13.9.1	Growth Factors	375
13.9.2	Erythropoietin	377
13.9.3	Amphetamine and Neurotransmitter Modulators	377
13.9.4	Anti-NoGo (IN-1)	377
13.9.5	Hedgehog	378
13.9.6	Stem Cells	378
13.10	Conclusions	379
	References	379

13.1 STROKE

13.1.1 Introduction and Symptoms

Stroke is the third leading cause of death in the United States, United Kingdom, and most developed countries after heart disease and cancer [1, 2]. It is a major cause of disability among adults and a major factor in late-life dementia. A stroke occurs when an artery bringing blood to the brain either becomes obstructed or ruptures and a part of the brain is deprived of oxygen. Without oxygen, nerve cells in the affected area of the brain are unable to function and then die within minutes. This results in loss of function in the part of the body which is controlled by these cells. The symptoms of stroke are easy to identify: sudden numbness or weakness, especially on one side of the body; sudden confusion or trouble speaking or understanding speech; sudden trouble seeing in one or both eyes; sudden trouble walking; dizziness; or loss of balance or coordination and/or sudden severe headache. Not all of these symptoms occur during a stroke and the symptoms depend on the location and amount of damaged tissue (Table 13.1). Sometimes, one or more of these warning signs may occur and then disappear and in this case may indicate a “ministroke,” also called a TIA (transient ischemic attack). Stroke symptoms occur suddenly (within minutes or hours) and often there are two or more symptoms.

TABLE 13.1 Major Symptoms Observed After Different Types of Stroke

Brain Region Affected	Primary Symptom	Other Symptoms
Right hemisphere	Paralysis on left side of body (left hemiplegia)	<p>Problems with spatial and perceptual abilities. For example, the stroke survivor may misjudge distances and fall or be unable to guide his or her hands to pick up an object.</p> <p>Impaired judgment and behavior. For example, he or she may try to do things that he or she should not attempt to do, such as trying to drive a car.</p> <p>Problems with short-term memory. Although he or she may be able to recount events from 30 years ago, may be unable to remember what he or she ate for breakfast that morning.</p>
Left hemisphere	Right hemiplegia	<p><i>Aphasia</i>—speech and language problems.</p> <p>Slow and cautious behavior, in contrast to the behavior of a right-hemisphere stroke survivor. They may need a lot of help to complete tasks.</p> <p>Memory problems similar to those of right-hemisphere stroke survivors. For example, he or she may have trouble learning new information and have poor short-term memory.</p>
Cerebellum	Balance/coordination	<p>Abnormal reflexes of head and torso</p> <p>Coordination and balance problems</p> <p>Dizziness, nausea, and vomiting</p>
Brain stem	Very severe as brain stem controls all of our involuntary “life support” functions, such as breathing rate, blood pressure, and heartbeat	<p>The brain stem also controls abilities such as eye movements, hearing, speech, and swallowing. Since impulses generated in the brain’s hemispheres must travel through the brain stem on their way to the arms and legs, patients with a brain stem stroke may also develop paralysis in one or both sides of the body</p>

13.1.2 Prevalence and Incidence

It is estimated that the *prevalance rate* for stroke is approximately 1 in 59 (1.69%), or 4.8 million people in the United States (Box 1). The *worldwide incidence* of stroke suggests that approximately 15 million people worldwide survive minor strokes each year [Cardiovascular Diseases—Prevention and Control, World Health Organization

(WHO), 2001–2002] [3–7]. In the 1960s and 1970s the prevalence of stroke started to fall, mainly because people were following advice to stop smoking, eat better, and exercise regularly [6, 7]. The incidence of stroke is markedly age related, but in the past 15 years the number of people having a stroke has remained constant as a consequence of an aging population. With improvement in health care (lower infant mortality, treatment of infections and other diseases) the prevalence of stroke is also increasing in developing countries.

BOX 13.1 PREVALENCE AND INCIDENCE STATISTICS

Prevalence and Incidence Statistics About Stroke

The estimated population of people who are managing stroke at any given time:

- Estimated 4.8 million in the United States, 2001 (American Heart Association, 2004).
- 2.4% of adults had ever had a stroke in the United States, 2002 [Summary Health Statistics for U.S. Adults, 2002, National Center for Health Statistics (NCHS), Centers for Disease Control (CDC)].

Incidence Statistics About Stroke

Annual diagnosis rate, or the number of new cases of stroke diagnosed each year:

- 3 million cases per year in the United States, 1994 (U.S. government statistics)
- Every year about 115,000 people in the United Kingdom have a stroke with a mortality of > 50%.
- 40,000–48,000 strokes occur annually in Australia (Australia's Health, 2004, Australian Institute of Health and Welfare).

13.1.3 Other Types of Stroke and Stroke-Related Brain Injury

Stroke also occurs due to hemorrhage (or bleeding into the brain parenchyma). Hemorrhagic stroke occurs in 20–30% of cases and is often due to an aneurysm (ballooning out and eventual bursting in the wall) of a weakened blood vessel. About a third of people who have bleeding in their brains die within 30 days, compared with about 8% of people who have a stroke because of a blocked artery.

Ischemic brain damage also occurs in many cases of traumatic brain injury (TBI), also called head injury, every year when a sudden trauma causes damage to the brain. TBI can result when the head suddenly and violently hits an object (e.g., a car crash) or when an object pierces the skull and enters brain tissue (e.g., a stab or bullet wound). Approximately 2 million patients in the United States attend hospital each year with a minor head injury, with 75,000 deaths and 75,000 left permanently disabled.

13.2 MECHANISMS OF ISCHEMIC BRAIN INJURY

In cerebral ischemia the brain is deprived of oxygen and glucose, adenosine triphosphate (ATP)-driven pumps fail to work, leading to a loss in ion homeostasis, depolarization, and release of neurotransmitters (lack of ATP also prevents glutamate reuptake and indeed the transporter may reverse under severe ischemic conditions). The excessive increase of extracellular glutamate following ischemia is thought to play a critical role in the development of neuronal damage [8–10]. Glutamate acts as an endogenous neurotoxin, and this led to the proposal of the excitotoxic hypothesis (for review see [11, 12]). Glutamate acting on N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) kainate receptors allows calcium to enter cells and entry can also occur via voltage-sensitive calcium channels. Glutamate can also act on metabotropic receptors leading to the production of diacylglycerol and inositol triphosphate, which activate enzymes and lead to the release of calcium from intracellular stores. The net calcium “overload” can activate several degradative enzymes (proteases, nucleases, phospholipases, NO synthase, etc.), and this can lead to the formation of free radicals and cell death. Thus, calcium-activated phospholipases catalyze the hydrolysis of membrane phospholipids and generally require calcium as a cofactor. In particular, phospholipases A2 and C can act by direct membrane breakdown or by generating toxic metabolites. Phospholipid breakdown can lead to the accumulation of lysophospholipids and free fatty acids such as arachidonic acid. Arachidonic acid can initiate a cascade of biochemical events leading to the production of prostaglandins, thromboxanes, and leukotrienes, and these are known mediators of inflammatory and allergic reactions. Metabolism of arachidonic acid by the lipoxygenase and cyclooxygenase pathways leads to the formation of harmful oxygen free radicals. Increased intracellular calcium can also activate proteases which sever the link between cytoskeletal network and the plasma membrane, cause disassembly of microtubules, and trigger proteolytic degradation of cytoskeletal components [13]. Calpains are Ca^{2+} -dependent proteases that degrade numerous cytoskeletal proteins. Calcium can also activate endonucleases (resulting in DNA fragmentation) and nitric oxide synthase (resulting in nitric oxide and peroxynitrite production), which also lead to neuronal death. In addition, studies have shown that apoptotic mechanisms contribute to cell death *in vitro* and damage following cerebral ischemia *in vivo*. Intervention with caspase inhibitors can provide protection against cell death in certain *in vitro* and *in vivo* model systems.

Neuroinflammation is another important contributing factor in ischemic brain injury and other neurodegenerative disorders [14]. It is well known that edema and brain swelling after brain injury contribute adversely to outcome and that anti-inflammatory therapy in stroke models can reduce tissue edema and improve outcome. However, the inflammatory response to injury, via the release of inflammatory mediators, can also directly damage the integrity of the brain. A summary of the many pathways contributing to ischemic brain injury is shown in Fig. 13.1. The whole process is also accompanied by alterations in gene expression, in both the ischemic core and the surrounding “penumbra,” where the pattern of cell death is both necrotic and apoptotic. It is likely that many of the regulated genes—switched on or off by an ischemic event—promote a return to homeostasis, but it is clear that many also contribute to the ensuing cell death. Some of the gene families that are activated include stress-related, mitogen-activated, adhesion molecule, cytokine, and

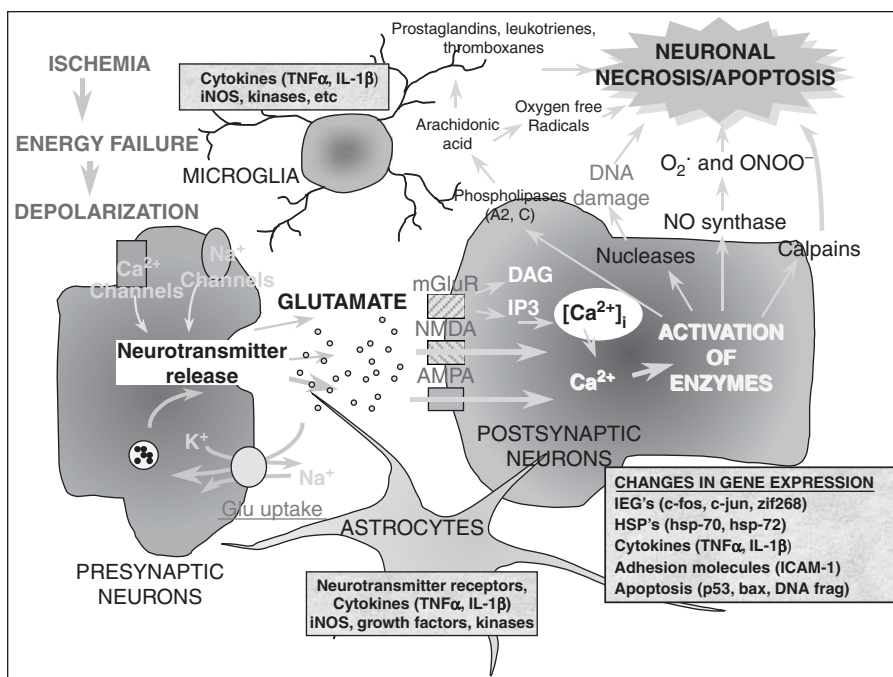


Figure 13.1 Potential mechanisms of ischemic brain injury. Following ischemia there is rapid depolarization, neurotransmitter release, and a cascade of Ca^{2+} -mediated enzyme activation and gene expression is set in motion. These pathways include stress and inflammatory pathways with downstream mediators such as p38 MAPK, iNOS, and cytokines. There is also marked activation of astrocytes and microglia. In addition the production of free radicals such as NO^{\bullet} , OH^{\bullet} , and ONOO^- can also contribute to cell death. Abbreviations: TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; Glu, glutamate; mGluR, metatropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DAG, diacyl glycerol; IEG, immediate early gene; IP3, D-myo-inositol (1,4,5)-triphosphate; HSP, heat-shock protein; ICAM, intercellular adhesion molecule-1; NO^{\bullet} , nitric oxide; OH^{\bullet} , hydroxyl radical; ONOO^- , peroxynitrite anion. (Modified from M. J. O'Neill et al., *Drug Discovery Today—Therapeutic Targets* 1, 59–67, 2004, with permission.) (See color insert.)

growth-factor-related genes and proteins, all of which have major roles in stress and inflammatory responses. The expression of many of the inflammation-related genes and proteins is altered for a considerable period after the initial insult to the brain. The most well described mediators of inflammation are the cytokine family [interleukin-1 (IL-1), interleukin-6 (IL-6), interferons, chemokines, etc.] and tumor necrosis factor alpha (TNF- α). Ischemic challenges increase the release of a number of cytokines both in vitro and in vivo [15], and many cytokines may contribute to neuronal cell death. Cytokine expression is regulated both at transcriptional and posttranscriptional levels. Many of these cytokines are produced as inactive precursors and are then subsequently cleaved to active molecules. For example, IL-1 β is cleaved by IL-1-converting enzyme (ICE, or caspase-1). The naturally occurring endogenous IL-1 inhibitor, IL-1 receptor antagonist (IL-1ra) has been shown to reduce brain damage in a transient middle cerebral artery occlusion (MCAO) model of stroke.

In summary, multiple pathways are contributing to ischemic brain injury, and an agent that targets (1) many pathways and (2) downstream events in the cascade would be a good choice as a potential neuroprotective agent.

13.3 ANIMAL MODELS OF GLOBAL AND FOCAL ISCHEMIA

The animal models used for preclinical stroke research fall into two main classes: (1) global ischemia and (2) focal ischemia. Global ischemia (two-vessel occlusion/bilateral carotid artery occlusion in gerbils, rats, and mice and four-vessel occlusion in rats) models mechanisms pertinent to cardiac arrest [16], while focal ischemia (MCAO in various species) models mechanisms pertinent to clinical stroke [17]. Focal infarcts in the middle cerebral artery (MCA) territory account for 25% of ischemic strokes in humans. It has been suggested that up to 80% of all strokes are the result of ischemic damage in the MCA area. For this reason, many of the models developed focused on this artery and most of these models are produced by either permanent or temporary occlusion of the MCA [17]. MCAO models produce consistent infarcts and can be modified to allow investigators control of the time of occlusion and the events that occur after reperfusion. Focal ischemia can be induced by electrocoagulation of a blood vessel, application of clips or ligatures, intraluminal monofilament, or injection of preformed clots and can be either transient or permanent (Fig. 13.2).

The choice of model depends on the mechanism being studied and the aspect of stroke one wants to mimic. The electrocoagulation (Tamura) model of permanent MCAO [18, 19] was widely used to study the neuroprotective actions of NMDA

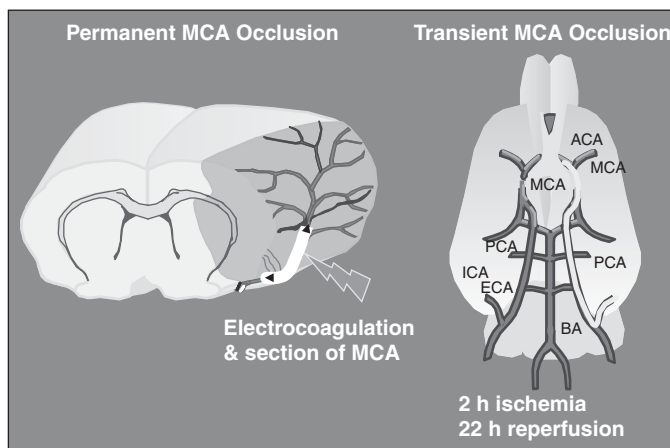


Figure 13.2 Two most commonly used rat models of focal ischemia. The Tamura model is produced by electrocoagulation of the MCA and is a permanent occlusion. The intraluminal model involves insertion of a coated monofilament into the internal carotid artery and advancing it until it blocks the origin of the MCA. The monofilament (yellow) can be left in place to provide permanent occlusion or removed after 60–120 min to allow reperfusion. Abbreviations: ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery; BA, basilar artery. (See color insert.)

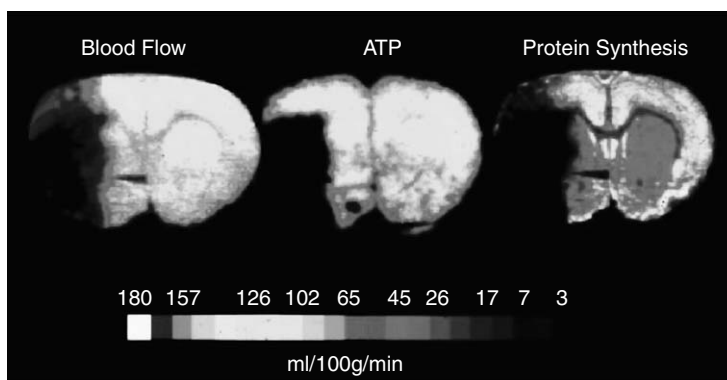


Figure 13.3 Illustration of ischemic penumbra. The coronal sections at the level of the striatum illustrate blood flow (left), ATP (middle), and protein synthesis (right) after 1 h of MCAO. There is an area of core damage with no blood flow that is surrounded by an area of brain tissue with reduced blood flow (penumbra). This area of brain is called the penumbra and in theory this tissue can be saved with a neuroprotective agent. (Original figure by Gunter Mies.) (See color insert.)

receptor antagonists (Fig. 13.2). However, in most stroke cases the blood vessel is blocked for a period of time and then the clot dissolves and blood flow is restored. It is likely that downstream pathways and those activated by reperfusion injury (such as free radicals and inflammation) are best studied in a transient model when blood flow is restored after a period (usually 60–120 min). The intraluminal monofilament model ([20, 21]; Fig. 13.2) is ideal for this, but the monofilament itself may damage the vessel wall and perhaps cause additional inflammation that may not be present in the clinical setting. For this reason it is now common practice to test for efficacy with a new intervention in at least two models.

The primary endpoint that was initially used in these models was histological assessment of brain damage. Methods were developed to section the brain at several stereotaxic levels and measure the area of damage and calculate the volume of damage (infarct volume). A neuroprotective drug would reduce the infarct volume (in cubic millimeters). The concept of the ischemic penumbra [22–27] is crucial to utilizing these models (Fig. 13.3). MCAO produced a core of damage in the areas of the brain directly perfused by the MCAO but also a surrounding area of penumbra where the tissue was hypoperfused and energy impaired. The penumbra has some blood supply from collateral vessels, and therefore this area of brain tissue can be salvaged using neuroprotective drugs.

13.4 GLUTAMATE RECEPTORS AND DEVELOPMENT OF NMDA RECEPTOR ANTAGONISTS

13.4.1 Glutamate and Glutamate Receptors

The amino acid L-glutamate is the major excitatory neurotransmitter in the mammalian brain and spinal cord [28]. Glutamate activates ligand-gated ion channels (ionotropic glutamate receptors, or iGluRs) and receptors coupled to second-messenger systems (metabotropic glutamate receptors, or mGluRs). The ionotropic receptors are divided

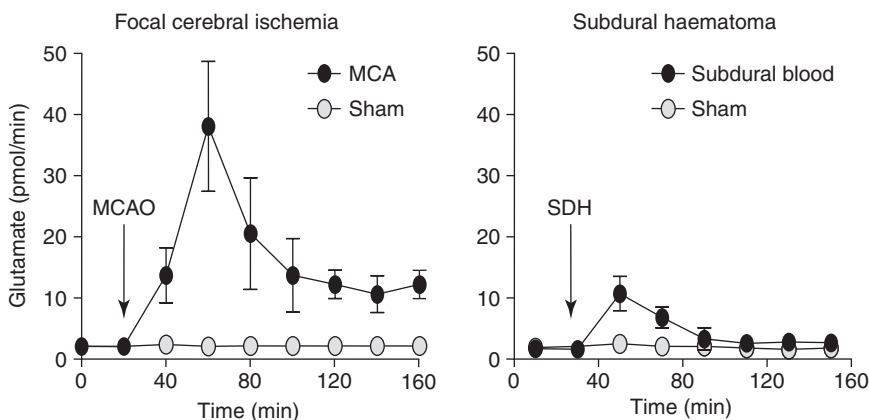


Figure 13.4 The Effects of focal ischemia or subdural hematoma on extracellular glutamate levels in rat brain (SDH, subdural hematoma).

into two distinct subtypes, namely, NMDA receptor and the AMPA and kainate receptor subtypes [29–31], while the metabotropic receptors consist of mGluR1–mGluR8 [31]. Early data indicated that glutamate and glutamate analogues (NMDA) were toxic to cells in culture. It was also observed that intracerebral (i.c.) or intracerebroventricular (i.c.v.) infusion of ibotenic acid or kainic acid produced local brain damage. Additional data indicated that the extracellular levels of glutamate were elevated after ischemia [32, 33] and hemorrhage (Fig. 13.4).

13.4.2 MK-801 : The “Gold Standard”

The most convincing data on the role of glutamate in ischemia came in the late 1980s as the first studies with NMDA antagonists in models of ischemia were reported. Early proof of concept studies with 2-amino-7-phosphonoheptanoic acid indicated that blockade of the NMDA receptor could provide neuroprotection against global ischemia [34]. However, after this and much of the other early data (dose response, dosing postocclusion, focal ischemia, etc.), excitement was generated with (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-iminemaleate (MK-801, Dizocilpine), which went on to be the gold standard or at least a reference standard in many preclinical laboratories. MK-801 is an orally active anticonvulsant and a potent and selective noncompetitive antagonist of NMDA responses in rat. MK-801 blocks the activated state of the NMDA receptor by an open-channel action. Systemic administration of MK-801 to rats confers protection against NMDA-induced neuronal degeneration. In 1987 Gill and co-workers at Merck Sharpe and Dohme in Essex tested MK-801 in the gerbil model of cerebral ischemia and found that it provided neuroprotective effects [35]. The pertinence of gerbil models to stroke and the extent to which drug-induced hypothermia may have contributed to neuroprotection have been the subject of much discussion.

The effects of NMDA antagonists (such as MK-801) in experimental models of focal cerebral ischemia are remarkably consistent, namely, the marked reduction in the amount of irreversible ischemic damage irrespective of the species, the model of cerebral ischemia, when the animals are sacrificed after the ischemic episode, whether

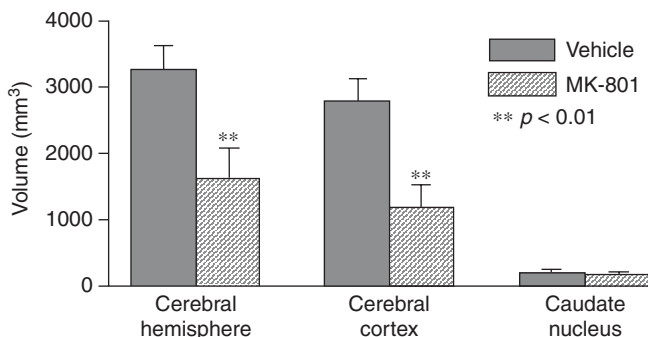


Figure 13.5 Protective effects of MK-801 in cat model of permanent MCAO. MK-801 was administered at 5 mg/kg i.v. 30 min prior to occlusion. MK-801 provided a 56% reduction in infarct volume when the median vehicle (3280 mm³) and MK-801 (1420 mm³) treated animals are compared. Temperature is regulated throughout this study [38].

ischemia is permanent or temporary and followed by reperfusion, the particular NMDA antagonists employed, and its site of action within the NMDA receptor complex [36–38]. Large-animal studies (e.g., cat) provided particularly compelling evidence with 50% reduction in volume of damage being observed with MK-801 pretreatment or where treatment is initiated 2 h after MCAO ([38]; Fig. 13.5).

However, the side-effect profile (psychotomimetic-like behavioral effects, learning and memory impairment [39, 40], and the production of vacuoles and neuronal damage in rat neocortical areas [41, 42], impacted adversely on the clinical development of MK-801 and other early NMDA antagonists. There was further confusion with the discovery that MK-801 caused hypothermia and led to the suggestion that MK-801 was providing neuroprotection by producing hypothermia [43]. In our preclinical studies (with the temperature controlled) MK-801 provided approximately 30–40% neuroprotection, and many other papers have confirmed neuroprotection with MK-801 in temperature-controlled studies. The published evidence that MK-801 is neuroprotective in focal cerebral ischemia is compelling and the compound has been evaluated by several independent laboratories (both academic and industry).

13.4.3 Other NMDA Antagonists

13.4.3.1 Studies with Other Competitive and Noncompetitive NMDA Antagonists.

Other competitive NMDA antagonists, such as D-(*E*)-4-(3-phosphonoprop-2-enyl) piperazine-2-carboxylic acid (D-CPP-ene, or SDZ EAA 494) and *cis*-4-phosphonomethyl-2-piperidine-carboxylic acid (CGS 19755, Selfotel), were also developed for treatment of stroke. As with many early efforts, Selfotel was initially shown to be beneficial in the gerbil model of global ischemia [44, 45], which companies often used as a more rapid *in vivo* filter/screen and then later studies demonstrated effects in rat and cat [46] models of focal ischemia. D-CPP-ene was also found to provide neuroprotective effects in rat models of cerebral ischemia [47, 48]. In a cat model of permanent MCAO a reduction of 64% was observed when D-CPP-ene was administered at 15 mg/kg before occlusion [49]. Detailed reviews of protective

effects of Selfotel [50] and D-CPP-ene [51] in animal models of cerebral ischemia and traumatic brain injury and in early clinical results have been published. Both molecules were progressed to the clinic and administered to stroke or trauma patients. Follow-up preclinical studies reported that Selfotel produced severe motor side effects at doses that protected in the mouse [52]. Interestingly, in the same study (\pm)-2-amino-*N*-(1-methyl-1,2-diphenyl)acetamide hydrochloride (Remacemide) was welltolerated and shown to have a therapeutic ratio of 10 [52]. Therefore, it appears remacemide and its active metabolite [(*S*)- α -phenyl-2-pyridine-ethanamine dihydrochloride] AR-R15896AR are well-tolerated NMDA antagonists. The pharmacology and neuroprotective effects of remacemide in various models of ischemia were published [53]. Like the parent molecule, AR-R15896AR is effective in rodent models of global and focal ischemia [54, 55] and was progressed to human trials.

Several other molecules have been examined preclinically, for example, CNS 1102 (aptiganel hydrochloride, or Cerestat) from Cambridge Neuroscience and NPS 1506 [3-fluoro- γ -(3-fluorophenyl)-*N*-methyl-benzenepropamine] from NPS Pharmaceuticals. CNS 1102 is an ion channel blocker, which was shown to have protective effects in models of cerebral ischemia [56] and progressed into clinical trials [57]. NPS 1506 is a noncompetitive NMDA receptor antagonist with moderate channel affinity. NPS 1506 is neuroprotective in rodent models of ischemic stroke [58], hemorrhagic stroke, and head trauma, with a 2-h window of opportunity. Neuroprotectant doses of NPS 1506 ranged from approximately 0.1 to 1.0 mg/kg, with peak plasma concentrations ranging from 8 to 80 ng/mL. Even at doses producing behavioral toxicity, NPS 1506 did not elicit MK-801-like behaviors and did not generalize to phencyclidine (PCP). In a phase I study, intravenous (i.v.) doses of NPS 1506 from 5 to 100 mg were well tolerated and provided plasma concentrations in excess of those required for neuroprotection in rodents. Adverse events at the 100-mg dose included mild dizziness and lightheadedness and mild to moderate ataxia [58].

13.4.3.2 Polyamine Site Antagonists of NMDA Receptor. Ifenprodil and its derivative (\pm)- α -(4-chlorophenyl)-4-[(4-fluorophenyl)methyl]-1-piperidineethanol (SL 82.0715, or Eliprodil) are phenylethanolamines, which are noncompetitive NMDA antagonists acting selectively at NR2B-subunit containing NMDA receptors and that have been shown to have neuroprotective activity in experimental models of ischemia. Eliprodil was reported to have neuroprotective effects in mouse [59] and cat [60] models of focal ischemia and was subsequently progressed to clinical studies [61]. A comprehensive review of the preclinical pharmacology and early clinical experience with Eliprodil was recently published by Carter and co-workers [62]. More recently, F. Hoffmann and La Roche have reported that Ro 63-1908, 1-[2-(4-hydroxyphenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol, is a novel subtype-selective NMDA antagonist that has been characterized in vitro and in vivo. Ro 63-1908 gave a dose-related neuroprotective effect against cortical damage in a model of permanent focal ischemia [63]. Maximum protection of 39% was seen at a plasma concentration of 450 ng/mL. There were, however, no adverse cardiovascular or central nervous system (CNS) side effects seen at this dosing level [63].

13.4.3.3 Glycine Site Antagonists of NMDA Receptor. Another target for neuroprotection is the glycine site of the NMDA receptor complex [64, 65]. Initial studies reported that 7-chlorokynurenic acid reduced ischemia-induced CA1 cell loss in the

gerbil [66, 67] and attenuated ischemia-induced learning deficits in rats subjected to global cerebral ischemia [68]. More recent studies have reported that newer glycine site antagonists such as (+ R)-HA-966 [3R-(+)-*cis*-4-methyl-HA966(L-687, 414), or (+)-3-amino-1-hydroxy-2-pyrrolidone] provide protection in a rat model of focal cerebral ischemia [69]. Other studies have reported that 5-nitro-6,7-dichloro-2,3-guinoxalinedione (ACEA 1021) and 5-nitro, 6, 7-dibromo-1, 4-dihydro-2, 3-guinoxalinedione (ACEA 1031) reduced cerebral infarct volumes after focal cerebral ischemia in rats [70]. In the early 1990s, Merck Sharp and Dohme reported the synthesis of potent and selective glycine antagonists derived from kynurenic acid [71], but further evaluation of these 4-substituted-3-phenylquinolin-2(1*H*)-ones at Merck led to compounds that were more potent in vitro activity but with reduced in vivo activity [71]. Another study indicated that binding to plasma proteins limits the brain penetration of many of these compounds [72], and for this reason none of these compounds progressed into the clinic. Glaxo Wellcome went on to report that [(*E*)-3[(phenylcarbamoyl)ethenyl]-4,6-dichloroindole-2-carboxylic acid sodium salt (GV150526A, Gavestinel), a related structure to the Merck compounds, reduced the infarct volume following MCAO and progressed this molecule into clinical trials. At Eli Lilly we found no neuroprotection with Gavestinel in a model of global cerebral ischemia [73]. We also failed to see any behavioral effects at high (200-mg/kg) doses of Gavestinel. Dawson and co-workers reported some protection with (+ R)-HA-966 but none with Gavestinel in a mouse model of focal ischemia [52]. These authors also failed to see any behavioral effects at doses of Gavestinel up to 300 mg/kg. It would appear from these studies that the compound fails to cross the intact blood-brain barrier (or does so at very low concentrations) and would not achieve the pharmacologically relevant doses required for clinical studies.

13.4.4 Clinical Data

As mentioned earlier, a number of NMDA antagonists entered clinical trials based on the preclinical data packages. In this section, we will summarize the outcome with Selfotel, Remacemide, and Cerostat (competitive and noncompetitive NMDA antagonists), Eliprodil (a polyamine site antagonist), and Gavestinel (a glycine site antagonist).

The competitive glutamate antagonist Selfotel entered clinical trials for stroke and head injury. Patients enrolled in neurotrauma trials are generally severely injured, such that they are unconscious and normally are sedated for intubation and ventilation. As a consequence, sedative or psychotomimetic effects of putative neuroprotective drugs rarely limit dosing, and this was the case with Selfotel. Within the head injury trials, doses that could be administered were much higher than for stroke. Within the phase IIa study of stroke patients, the use of Selfotel was associated with sedation, agitation, confusion, and hallucinations [74]. These symptoms generally came on within an hour or two of administration and persisted for two to three days. Phase III trials moved forward with the maximum tolerated dose, of 1.5 mg/kg. Even so, marked adverse effects were frequently encountered. Phase III trials were abandoned early after safety analysis identified a significant increase in early mortality associated with Selfotel use [75].

The low-affinity NMDA antagonist Remacemide was tested in a single phase IIa trial in stroke [76]. Mortality was unaltered and death/dependency data were encouraging, but it was recognized that the active metabolite was formed too slowly to be of likely benefit in acute stroke. Two phase II trials with the sister compound,

ARR-15896AR, were undertaken but only one has been published to date [77]. Mortality was unaltered, but death/dependency seemed to be increased, perhaps due to an imbalance in prognostic variables at baseline.

The noncompetitive glutamate antagonist, Cerostat/Aptiganel, appeared to have more favorable pharmacokinetic properties. The CNS effects of the drug were apparent within minutes of commencing infusion, confirming rapid penetration to the brain, and in the event of excessive side effects, infusions could be discontinued with resolution of the effects after at most a few hours [78, 79]. Nevertheless, a similar constellation of side effects was encountered with Aptiganel, namely sedation, nystagmus, hallucinations, and in extreme cases a transient catatonic state. These symptoms limited dosing once again, but plasma concentrations in the range of 10–12 ng/mL were achievable in stroke patients, on the threshold of those that were associated with neuroprotection in rat models of stroke [80, 81]. Aptiganel was explored in a small phase IIa study in stroke patients before proceeding to a phase IIb/III trial. This trial was abandoned at the first planned interim analysis on the basis of apparent futility.

The polyamine site antagonist Eliprodil entered development for stroke. The dose chosen for efficacy trials was well tolerated but had been selected partly on the basis of its freedom from QT prolongation, a potential sideeffect observed in preclinical testing [61]. Again, there is a reasonable probability that the dose was subtherapeutic. In any event, the phase III trial testing Eliprodil within 8 h of onset of acute stroke was terminated when sequential (interim) analysis suggested futility.

The glycine site antagonist Gavestinel was pursued through a full clinical development program. The preclinical data available on Gavestinel were entirely derived from the sponsoring company [82]. These had been presented but not published in peer-reviewed journals at the time [83]. The drug appeared to have efficacy when administered up to 6 h after stroke onset in animals and certainly was well tolerated by stroke patients [84]. Target plasma concentrations were achieved and maintained for 72 h without major problem; higher maintenance doses were associated with some reversible disturbances of liver function tests [85]. These were attributed partly to competition for binding sites rather than to any direct hepatotoxic effect. Phase IIb trials examined Gavestinel within 12 h of stroke onset [86, 87]. Gavestinel proceeded into two large phase III trials, both of which were completed on schedule. The average time to treatment was under 5 h in the international trial and just over 5 h in the American trial. Baseline balance on prognostic factors was excellent in both trials, but the outcome in both trials was unequivocally neutral [88, 89]. As mentioned earlier, the penetration of Gavestinel into brain tissue was substantially poorer than initially believed with the cerebrospinal fluid level being only 0.04% of the plasma level in humans [88].

13.5 SUMMARY OF ISSUES WITH PRECLINICAL STROKE STUDIES USING NMDA ANTAGONISTS

13.5.1 Side Effects and Other Issues with Development of NMDA Antagonists

The NMDA antagonists are examples of compounds that worked in animal models of cerebral ischemia but had difficulties in the clinic. Many of the problems could have been prevented if the early preclinical studies were conducted with more rigor.

For example, in many cases critical parameters such as temperature and blood pressure were not monitored. Likewise, pharmacokinetic studies would have allowed experiments that gave the correct dose, dosing protocol, and duration of coverage to be conducted in the preclinical ischemia models. More importantly, side effects (sedation, respiratory depression) were overlooked. In our experience, many ischemia studies utilized doses that produce side effects (sedation, ataxia, respiratory depression, hypothermia, etc). Many of the pharmaceutical companies that developed these molecules had preliminary tertiary screening that included mouse behavior, Irwin screens, locomotor studies, and other observational testing and all molecules that progressed to the clinic had to undergo preclinical safety and toxicological studies. Indeed NMDA antagonists were/are routinely used to produce hyperactivity and stereotypic behaviors [39, 90] and increased *c-fos* and *hsp-70* expression [90, 91] that can be reversed by antipsychotics [90, 91]. In addition, NMDA antagonists produce memory impairment [40] and higher doses of early NMDA antagonists produced neuronal vacuolization [41, 42]. Thus, acute and chronic administration of Selfotel was found to produce marked CNS effects and high doses, 10–100 mg/kg i.v., produced vacuolization [46]. D-CPP-ene was evaluated in mice and rats. In a “primary observation test” in mice D-CPP-ene reduced locomotor activity and reduced rearing and produced ataxia. Cerestat produced stereotypic behavior, ataxia, and sedation at doses of 1 mg/kg [57]. While it is always a case of what is the margin of safety and no drug is without some side effects, all of the effects with the NMDA antagonists were observed at doses that were subsequently required neuroprotection. The exception appears to be Remacemide, where much higher doses were required to produce overt behavioral changes. There was also a lack of studies correlating dose with neuroprotective plasma and brain levels. This would have provided valuable information and ensured that expensive clinical trials (i) were never initiated or (ii) tested the desired hypothesis (achieved neuroprotective plasma levels, an issue that plagued the clinical development of these agents). Another consideration often ignored is (iii) that none of the animal models used can mimic all aspects of human stroke. Therefore positive effects in more than one model are desirable. Furthermore, (iv) NMDA antagonists fail to prevent damage to white matter (axons and oligodendrocytes) [92] and this is a significant part of the stroke pathology (Fig. 13.6).

13.5.2 Time Window and Other General Issues

Other issues are more general and relate to most preclinical stroke work. For example, very few of the earlier studies were carried out to fully assess the time window of neuroprotection in rodent studies. Thus, agents that protected immediately or 30 min after ischemia in animals were advanced to clinical studies where patients arrived 3–12 h after stroke. There is some debate in the literature as to the time course of progression of ischemic brain damage in rat and humans. Many studies in rats indicate that the ischemic pathology occurs rapidly and metabolic mapping and functional magnetic resonance imaging (fMRI) studies show reduced perfusion and increased water content at 3 h and in some cases investigators used 6 h as an endpoint where histological damage has occurred. It is thought that in humans the progression of stroke is somewhat slower and that damage is occurring at 6 h in the penumbra, and some studies suggest that this may even be at 9–12 h. Mismatch

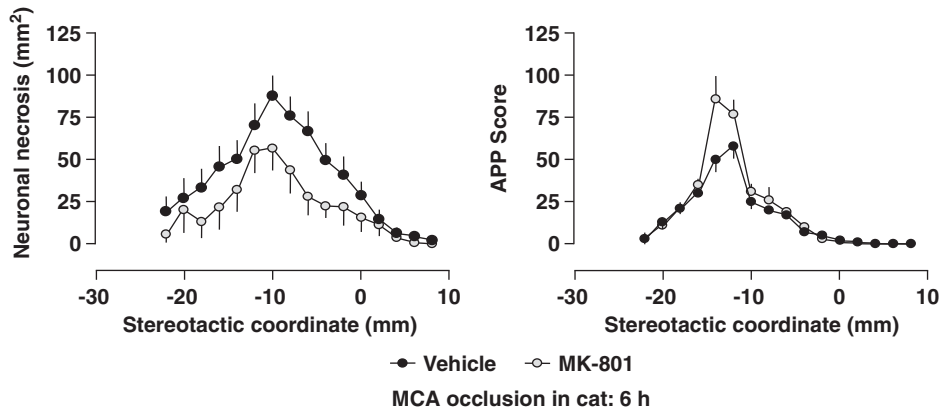


Figure 13.6 Illustrates that NMDA antagonist MK-801 reduces neuronal perikaryal damage (left panel) but not axonal damage (right panel) in brain after MCAO in cat [92].

imaging studies allow perfusion deficits to be compared with T2-weighted (T2W) fMRI deficits, and the tissue with low perfusion (penumbra) that does not progress to infarct is considered salvageable. In rat models it is difficult to get protection with any agent 2–3 h after occlusion. Therefore, agents which showed efficacy 3 h after occlusion (such as Cerovive) are attractive candidates to evaluate in human stroke.

More difficult to address is the fact that preclinical studies were carried out under controlled conditions using young animals, whereas human strokes occur in the aged population and are very heterogeneous in nature. Further aspects relate to the type of stroke (lacunar, small–large MCA) and in particular clinical trial design and rating scales. The purpose of animal models is not to replicate human disease but to investigate mechanisms which are pertinent to human disease. The crucial issue is often neglected when a drug makes the transition from preclinical to clinical development.

13.6 OTHER GLUTAMATERGIC APPROACHES

13.6.1 AMPA Receptor Antagonists

13.6.1.1 Studies with NBQX and Related Quinoxalinediones. Neuroprotective effects following pretreatment with the competitive AMPA receptor antagonist 3-dihydroxy-6-nitro-7-sulfamoyl-benz[f]quinoxaline (NBQX) were first reported in gerbil global ischemia by Sheardown et al. [93]. Delayed treatment was also effective in models of global ischemia [94, 95]. NBQX also reduced infarct volume following focal ischemia in rats [96, 97]. However, the older quinoxalinedione AMPA antagonists, such as NBQX, have low solubility at physiological pH and precipitate in kidneys and cause nephrotoxicity [98]. NBQX was administered to healthy volunteers, but clinical development was stopped due to the low solubility and the risk of formation of crystals in the kidney tubules [99]. Newer molecules, such as 6-(1-imidazolyl)-7-nitroquinoxaline-2,3-(1*H*,4*H*)-dione monohydrochloride (YM-90 K) and [2,3-dioxo-7-(1*H*-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxaliny] acetic acid monohydrate (YM-872), were later

described. YM-872 appears to give robust neuroprotective effects in models of focal ischemia without the side-effect limitations [100, 101]. Further studies indicated that introduction of a phosphonate group improved the solubility of quinoxalinediones while maintaining activity at AMPA receptor subtypes [102]. The novel phosphonate quinoxalinedione derivative [1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl] methyl phosphonate (ZK200775) had a long therapeutic window in a rat model of permanent focal ischemia and was advanced to the clinic [103]. Neurosearch reported that another quinoxalinedione, SPD 502 [8-methyl-5(4-(*N,N*-dimethylsulfamoyl)phenyl)-6,7,8,9-tetrahydro-1*H*-pyrrolo[3,2-*h*]-isoquinoline-2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime] (10-mg/kg bolus injection followed by a 10-mg/kg/h infusion for 2 h) resulted in a highly significant protection against the ischemia-induced damage in the hippocampal CA1 pyramidal neurons [104]. In focal cerebral ischemia, AMPA receptor antagonists such as SPD 502 reduce damage to neuronal cell bodies but crucially also protect neuronal axons and oligodendrocytes [105]. These effects contrast with NMDA antagonists which do not protect neuronal axons or oligodendrocytes in focal cerebral ischemia.

13.6.1.2 GYKI 52466 and Related 2,3-Benzodiazepines. 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466) and other 3-*N*-substituted 2,3-benzodiazepines have neuroprotective effects in global [106–108] and focal [98, 109] cerebral ischemia. 2,3-Benzodiazepines are selective, noncompetitive AMPA receptor antagonists with little effect on kainate receptors [110]. Various substitutions within the 2,3-benzodiazepine molecule resulted in more potent inhibitors of AMPA receptors (LY300164, LY300168)¹ in vitro [110, 111] and in vivo [108] and consequently in more effective neuroprotectants.

13.6.1.3 Decahydroisoquinolines. A distinct series of decahydroisoquinolines that included highly soluble, competitive AMPA receptor antagonists were also described [112, 113]. Among them, systemic administration of (3*SR*,4*aRS*,6*RS*,8*aRS*)-6-[2-(1*H*-tetrazol-5-yl)-ethyl]-1,2,3,4*a*,5,6,7,8*a*-decahydroisoquinoline-3-carboxylic acid (LY215490) was shown to reduce infarct volume after focal ischemia in rats [114]. The active isomer, (3*S*,4*aR*, 6*R*, 8*aR*)-6-[2-(1(2)*H*-tetrazole-5-yl)-ethyl] decahydroisoquinoline-3-carboxylic acid (LY293558), was neuroprotective in focal ischemia in both the cat [115] and rat [116] and in global ischemia in the gerbil [117]. This compound was put into development but never given to stroke patients because of dose-limiting side effects.

13.6.1.4 Summary of Issues with Preclinical Stroke Studies Using AMPA Antagonists. AMPA antagonists may be good candidates for clinical development as they lack the psychotomimetic action and neurotoxicity observed with NMDA antagonists. These compounds do produce sedation and ataxia, but they may protect astrocytes and oligodendrocytes as well as neurons (Fig. 13.7). However, until recently, none of the AMPA antagonists mentioned above have been suitable to progress into human stroke patients. ZK200775 was eventually progressed to phase IIa but then abandoned

¹LY00164, (–) enantiomer of (±) GYKI 53405, (–)-3-*N*-acetyl-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; LY300168, GYKI 53655, (±)-3-*N*-methylcarbamyl-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine.

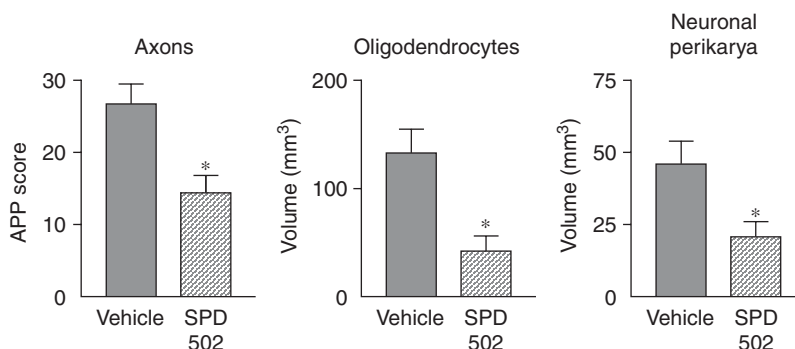


Figure 13.7 Illustrates that AMPA antagonist SPD 502 reduces damage to axons, oligodendrocytes, and neurons in brain after MCAO in rat. Data from [105].

(results not reported), and so this hypothesis remains to be tested in a phase III clinical trial. The issue with earlier compounds such as quinoxalinediones was solubility and nephrotoxicity. The other problems mirror that of NMDA antagonists and many earlier AMPA antagonists that produced ataxia, sedation, and respiratory depression. Newer molecules appear to show increased solubility and selectivity for various iGluR subunits and seem to produce more robust effects in animal models of ischemia.

13.6.2 Kainate Receptor Antagonists

Various 6-substituted decahydroisoquinolines have profiles of activity at NMDA-, AMPA-, and kainate-preferring receptors. Pharmacological studies with LY293558 indicated that the compound was actually a mixed AMPA and iGlu5 kainate receptor antagonist [110], and other compounds in the series, for example, (3*S*,4*aR*,-6*S*,8*aR*)-6-[2-(1*H*-tetrazol-5-yl)methoxymethyl]-decahydroisoquinoline-3-carboxylic acid (LY377770), were more selective iGlu5 antagonists [116–118]. When members of this series of decahydroisoquinolines, with various antagonistic profiles at NMDA, AMPA, and iGlu5 receptors, were studied in the gerbil global ischemia model, LY377770 provided the greatest neuroprotection [117], raising the prospect that iGlu5 antagonists may be effective neuroprotectants. LY377770 is neuroprotective in a rat model of focal ischemia [116]. LY377770 also provided a robust blockade of ischemia-induced glutamate release *in vivo* and had a longer time window and better side-effect profile than the more “AMPA-like” parent molecule, LY293558 [116]. Such results suggest that this target warrants further investigation.

13.6.3 Metabotropic Glutamate Receptors (mGluRs)

The advent of molecular biology and developments in signal transduction have spurred new efforts to modulate glutamate transmission in the CNS. The metabotropic family of glutamate receptor are G-protein-linked receptors, and in the late 1990s a number of new ligands for these receptors were described [119, 120], allowing further investigation of the proposed role of mGluRs in aspects of neurodegeneration [121–123]. As mGluRs play more of a modulatory role, it is possible that drugs acting on these receptors would be devoid of many of the side effects that plagued ionotropic ligands.

To date, eight subtypes of the G-protein-coupled mGluRs have been cloned and classified into three groups according to their second-messenger association, sequence homology, and agonist selectivity [120, 124]. Group II (mGlu2 and 3) and group III (mGlu4, 6, 7 and 8) mGluRs are negatively coupled to adenylyl cyclase and thought to act as presynaptic autoreceptors, regulating glutamate transmission [125]. Recent evidence indicates that mGlu3 receptors are also expressed by astrocytes and glia [126].

Activation of group II mGluRs is reported to protect neurons against excitotoxic degeneration by the inhibition of glutamate release [122, 127]. In support of this idea, the selective group II mGluR agonist LY354740 (2-aminobicyclo[3.1.0]hexane 2,6-dicarboxylate) [128] reduces veratradine-evoked striatal amino acid release [129] and field excitatory postsynaptic potentials (fEPSP) in rat hippocampal slices [130].

Although reduction of glutamate release is an attractive hypothesis, there is a body of evidence in vitro that suggests this may not always account for the neuroprotective activity of group II mGluR agonists. For example, Bruno et al. (1997) [131] have suggested that astrocytes, following exposure to group II mGluR agonists, produce a heat-sensitive factor in the culture medium, which itself has neuroprotective properties in cortical cells. More recently, this group has reported that TGF- β 1 (transforming growth factor- β 1) and TGF- β 2 were released from astrocytes exposed to group II mGluR agonists and that antibodies that neutralized the actions of TGF- β 1 or TGF- β 2 prevented the neuroprotective effects of the group II agonists DCG-IV [(2S, 1'R, 2'R, 3'R)-2-(2, 3-dicarboxycyclopropyl) glycine] and 4C3HPG (4-carboxy-3-hydroxy phenylglycine) in cultured cortical neurones [132].

Group II mGlu agonists are also reported to be neuroprotective in vivo [133, 134], but earlier compounds were not very selective. A more selective group II agonist, LY354740, has been shown to provide some neuroprotection in gerbil global ischemia [135] but none in rat focal ischemia [136]. Recently, a more potent and highly selective group II agonist, LY379268 (1R,4R,5S,6R-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate), has been discovered which has effective concentration EC_{50} values of 2.69 ± 0.26 and 4.48 ± 0.04 nM at human mGluR2 and mGlu3 receptors, respectively [137]. LY379268 provided neuroprotection against NMDA-mediated cell death in vitro [138] and almost complete protection against CA1 hippocampal damage after global ischemia in gerbils (Fig. 13.8) but failed to show neuroprotection against focal ischemia in the rat [139, 140]. These data suggest that perhaps this class of agent is more effective in situations of delayed neuronal injury, as is observed in global ischemia and less effective against acute necrosis and inflammation that are present in focal ischemia. The lack of effects in focal ischemia are a concern, as these models are more pertinent to clinical stroke.

The role of group I receptors in ischemia has also been studied. (S)- α -Methyl-4-carboxy-phenylglycine (MCPG, mGluR 1 and 5 antagonist), 1-aminoindan-1,5-dicarboxylic acid (AIDA, mGluR 1a specific antagonist), and 2-methyl-6-(phenylethynyl) pyridine (MPEP, mGluR 5 antagonist) were evaluated in a gerbil model of ischemia [141]. The authors reported protective effects with MCPG and MPEP, but only a minor effect with AIDA. In other studies, AIDA and (S)-(+)-2-(3'-carboxybicyclo[1.1.1]pentyl)-glycine (CBPG), another mGluR1 antagonist, were also reported to have protective actions in murine cortical cell cultures and rat organotypic hippocampal slices exposed to oxygen glucose deprivation (OGD) and in vivo, following transient global ischemia in gerbils [142, 143]. Furthermore, studies indicating that MPEP is protective in a rat focal ischemia model have been reported [144]. It is not clear if the protective actions of MPEP

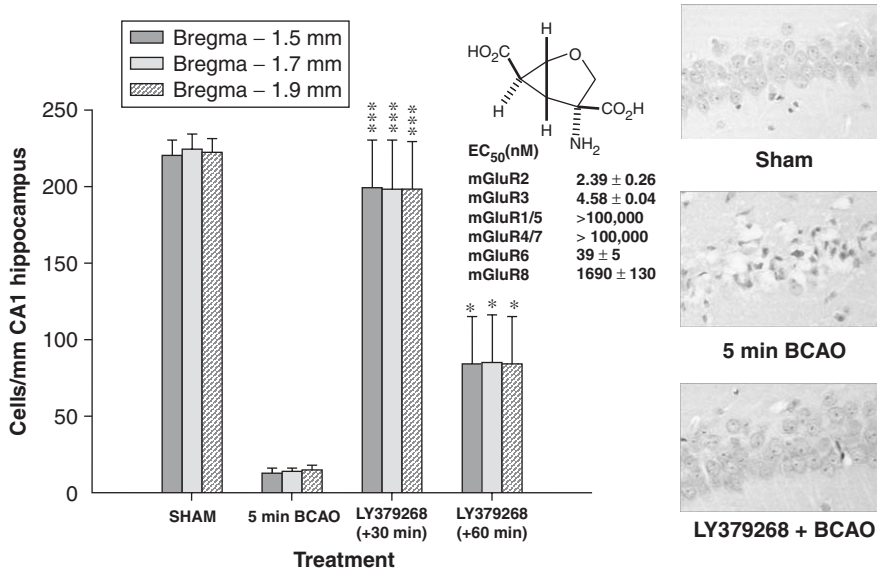


Figure 13.8 Effects of LY379268 (10 mg/kg i.p.) administered 30 or 60 min after occlusion on neuronal density at three stereotaxic levels in CA1 region of hippocampus five days after surgery. Histological results are expressed as mean \pm SEM viable cells/mm CA1 hippocampal region ($n = 8$ animals per group). Five minutes of BCAO produced severe damage to the CA1 region ($p < 0.0001$) while LY379268 produced protection when administration was initiated 30 min after ($p < 0.001$) or 60 min after ($p < 0.01$) occlusion (Student's t -test). The structure and mGluR selectivity profile of LY379268 are also inset. (See color insert.)

are solely mediated by mGluR5 antagonism as MPEP appears to have associated NMDA receptor activity [145].

In our opinion the overall side-effect profile of mGluR ligands looks much more favorable than that of ionotropic glutamate antagonists. However, the preclinical data to date are not robust or extensive. The magnitude of the effects and time window in focal ischemia models does not support clinical development of these agents for acute ischemic injury.

13.6.4 Glutamate Transporters

There are also reports that suggest that a major source of the glutamate released in severe ischemia comes from a reversal of glutamate transporter [146]. Therefore, inhibition of these transporters may also be a possible intervention for treating brain injury. It is possible that because many of the transporters are localized on astrocytes and glia, blocking these transporters would not alter normal synaptic transmission and perhaps not have the overt behavioral effects of classical glutamate antagonists.

Glial (GLT-1 and GLAST) and neuronal (EAAC1) high-affinity transporters mediate the sodium-dependent glutamate reuptake in mammalian brain. Their dysfunction leads to neuronal damage by allowing glutamate to remain in the synaptic cleft for a longer duration. GLT-1 has been reported to be downregulated prior to cell death following global ischemia in gerbil [147]. Time course studies of GLT-1 expression in the rat indicated that the reduction in GLT-1 expression evolved

concomitantly with the degeneration of CA1 pyramidal cells, and it may contribute to the severity of CA1 pyramidal cell loss [148]. Gottlieb et al. (2000) reported altered expression of the glutamate transporter EAAC1 in neurons and immature oligodendrocytes after transient forebrain ischemia in the rat [149]. Pharmacological studies using DL-threo- β -benzyloxyaspartate (DL-TBOA), a newly developed competitive, nontransported blocker of the EAAT 1–3 transporters, have also been reported [150]. Phillips and co-workers (2000) evaluated changes in the extracellular levels of amino acids, glucose, and lactate in cerebral cortical superfusates during four-vessel occlusion-elicited global cerebral ischemia using a cortical window technique [150]. Results indicated that reversed transport, primarily from glial cells by the EAAT 2 carrier, is responsible for a substantial (42 and 56%) portion of the ischemia-evoked increase in extracellular glutamate and aspartate levels, respectively.

Published data are however somewhat confusing, with antisense knockdown of the glial glutamate transporter GLT-1 reported to exacerbate transient focal cerebral ischemia-induced neuronal damage in rat brain [151] while gene deletion of GLT-1 augments brain edema after transient focal cerebral ischemia in mice [152]. The model used, the severity of the ischemic insult, and the timepoints evaluated are clearly important variables. More recent studies suggest that GLT-1 takes up extracellular glutamate to protect neurons in the early stage of ischemia and then releases glutamate, triggering acute neuronal death, when ischemic conditions are elongated [153]. The function of GLT-1 may change from neuroprotective to neurodegenerative during ischemia. In general, it would appear that inhibiting glutamate transporters may be detrimental under physiological or mild ischemic conditions, while, in contrast, inhibiting these transporters under severe ischemic insults is beneficial.

13.7 UPSTREAM AND DOWNSTREAM NEUROPROTECTIVE APPROACHES

13.7.1 Glutamate Release Inhibitors: Calcium and Sodium Channel Blockers (Up-stream Approaches)

As mentioned in Section 13.1, there are multiple mechanisms that contribute to ischemic injury. Other approaches to prevent brain injury included attempts to inhibit glutamate release. These approaches in general utilized Ca^{2+} or Na^{+} channel blockers.

13.7.1.1 Neuronal Calcium Channel Blockers. Early studies with synthetic conopeptides indicated that a single bolus intravenous administration of ω -conotoxin MVIIA (SNX-111), which blocks N-type calcium channels, provided protection when administered as late as 24 h after the ischemic insult [154]. Similar effects were observed in two other studies [155, 156]. ω -Conotoxin MVIIA has also been found to be highly effective in reducing the neocortical infarct volume in rat models of focal ischemia, both when administered during the occlusion [157] and after the ischemic episode [158].

One major drawback of conotoxins is that they are large peptide molecules, which do not cross the blood–brain barrier efficiently. Consequently, they have to be administered at high doses that often yield peripheral side effects such as hypotension (which exacerbates brain damage). However, neuroprotective effects with smaller nonpeptide compounds that inhibit neuronal calcium channels have been described.

For example, NNC 09-0026 [159, 160], SB 201823-A [161, 162] and NS-649 [163] have protective actions in animal models of cerebral ischemia.² We have evaluated all of the above small molecules in our models, and it is clear that these compounds provide only minimal protection and have a short time window. More recently, Eli Lilly has reported that another molecule, *N*-butyl-[5,5-bis-(4-fluorophenyl)tetrahydrofuran-2-yl]-methylamine hydrochloride (LY393615), has neuroprotective actions in global and focal cerebral ischemia [164].

13.7.1.2 Sodium Channel Blockers. Several investigators have also examined the role played by sodium channel blockers in ischemic brain injury [165–167]. Riluzole (Rilutek, 2-amino-6-trifluoromethoxy-benzothiazole) was protective after 6 min bilateral carotid artery occlusion (BCAO) in the gerbil [168]. Later studies have indicated that other sodium channel blockers such as lamotrigine [169] and BW619C89 [4-amino-2-(4-methyl-1-piperazinyl)-5-(2,3,5-trichlorophenyl) pyrimidine, sipatrigine [170, 171] are protective in models of cerebral ischemia. Another molecule, (f)-(S)-1-(4-E(2-benzothiazolyl-(methyl)-amino)piperidyl)-3-(3,4-difluorophenoxy)-2-propanol (lubeluzole, or Prosynap), was also shown to block ischemia-induced increase in glutamate [172] and was an effective neuroprotectant in animal models of focal ischemia [173, 174]. Although lubeluzole is a sodium channel blocker, the molecule also inhibited the signal transduction pathways of nitric oxide, and it was suggested that this may in fact be the mechanism of action [175, 176]. As with calcium channels, blockade of sodium channels has not been very successful, and neuroprotective doses often produced cardiovascular side effects, including effects on QT interval. However, sipatrigine and lubeluzole have been evaluated in acute stroke patients and will be discussed later in this chapter.

One of the major issues with both calcium and sodium channel blockers was the fact that these channels (1) perform key physiological functions and (2) are activated almost immediately after occlusion. The lack of energy to the brain produces depolarization and flux of ion through these channels and initiates glutamate release. These receptors/channels may indeed therefore be critical upstream targets that help initiate a cascade of secondary events. However, it is not likely that these channels would give any window of opportunity to intervene except where there are recurrent multiple ischemia episodes. Other major concerns with inhibiting these channels are the potential side effects (e.g. tremor and convulsions associated with blocking calcium channels and altered QT interval/heart beat with sodium channel blockers).

13.7.2 Anti-Inflammatory Agents, Antioxidants, Caspase and Other Antiapoptotic Approaches (Down-Stream Approaches)

As mentioned in Section 13.2, it is now known that multiple pathways contribute to ischemic brain injury. This knowledge has resulted in new neuroprotective strategies via many of the downstream (secondary) pathways. These can broadly be categorized into anti-inflammatory agents (cytokine inhibitors, chemokine inhibitors, p38

²NNC 09-0026, (–)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethylphenoxy)-methyl]piperidine dihydrochloride; SB 201823-A, 4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidine hydrochloride; NS 649, 2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole.

inhibitors, antiadhesion molecules, nitric oxide synthase inhibitors), antioxidants (free-radical scavengers, lipid peroxidation inhibitors, and nitric oxide synthase inhibitors), and anti apoptotic approaches (caspase inhibition). Many of these target mechanisms would appear to occur as a response to the primary insult in the penumbra of the stroke and therefore may offer a greater time window of opportunity for intervention. Several other agents (leukotriene inhibitors, statins, nonsteroidal anti-inflammatory agents) may also be important but will not be covered in the present review.

13.7.2.1 *Inflammatory Pathways.* Many of the genes that are altered by stroke are immediate responses to brain injury and may be intrinsic neuroprotective mechanisms or processes which limit the spread of the lesion. However, some of the gene families that are activated include stress-related, mitogen-activated, vascular, glial, and growth-factor-related genes and proteins and have major roles in stress and inflammatory responses. The expression of many of the inflammation-related genes and proteins is altered for a considerable period (hours to days) after the initial insult to the brain. In addition, many of these genes and proteins contribute to the processes involved in brain repair and response to injury, and could therefore provide targets for drug intervention. The best described mediators of inflammation are the cytokine family (IL-1, IL-6, interferons, chemokines, etc.) and TNF α .

13.7.2.2 *Cytokine Inhibition.* The cytokine family of polypeptides include TNF α , interleukins, chemokines, growth factors, and interferons and have been widely implicated in immune activation and inflammation [15, 177]. Many of these cytokines are produced as inactive precursors and are then subsequently cleaved to active molecules. For example, IL-1 α and IL-1 β are cleaved by ICE (caspase-1) and pro-TNF α is cleaved by the enzyme TNF α convertase. Other important CNS cytokines include IL-2, IL-3, IL-10, IL-6, and transforming growth factor beta (TGF β) [178].

The biology of the family is extremely complicated and the number of family members, signaling pathways, and biological effects are varied. Thus, the cytokines are attractive targets, but as some appear to act as “pro-inflammatory” and others “anti-inflammatory”, a clear understanding of the downstream effects of inhibiting each pathway is required [177]. In addition, even a single cytokine can have different effects depending on the model system used. Thus, for example, TGF β has been reported to be neuroprotective in some ischemic models but enhances damage in others.

The role of the cytokine IL-1 in stroke has largely been elucidated using the naturally occurring cytokine receptor antagonist (IL-1ra) [177, 179]. Attempts to discover small-molecule IL-1 receptor antagonists has been hampered by the nature of the large protein–protein interactions involved. Some progress has been disclosed in the form of a 15-amino-acid peptide antagonist [180] and subsequent peptidomimetic piperazine derivatives [181]. However, the current strategy for targeting IL-1 in stroke appears to be the inhibition of IL-1 processing via caspase-1 (ICE) inhibition. Evidence for a role for ICE inhibition in reducing ischemic brain injury has come from the use of ICE knockout mice [182] and the i.c.v. administration of peptidic ICE inhibitors [183]. Recently, some small-molecule inhibitors of TNF α convertase (TACE) have been discovered and found to be highly effective in blocking lipopolysaccharide (LPS)–induced TNF α release in vivo. Researchers at Bristol Myers Squibb have recently published the protective effects of a small-molecule

TACE inhibitor in a rat model of focal cerebral ischemia confirming the potential of this approach [184].

13.7.2.3 Chemokine Inhibition. Chemokines are a large family of small (~ 10 -kDa) structurally related peptides that were originally shown to be important in regulation of immune responses and migration of leukocytes [185, 186]. Chemokines are expressed at low levels in astrocytes, microglia, and neurons and are induced by inflammatory conditions. For example, the expression of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 α (MIP-1 α) is induced in the rat brain after peripheral lipopolysaccharide [187] and focal ischemia [186, 187].

The potential for the treatment of stroke by the direct antagonism of chemokine receptor binding has been demonstrated through the use of monoclonal antibodies and high molecular-weight peptidic antagonists [186]. Some of the specific mediators that appear to be involved in the progression of ischemic damage are the CC chemokines MCP-1 [188] and MIP-1 α [189] and IL-8 from the CXC class [190]. In recent years, there has been good progress made in identifying small molecule chemokine antagonists with compounds beginning to enter clinical development. However, the effective use of small molecules in preclinical stroke models has yet to be demonstrated.

13.7.2.4 p38 Inhibition. Another inflammatory pathway that has been studied recently is the p38 mitogen-activated protein kinase (MAPK) pathway [178]. This pathway can be activated by mitogens, environmental stress, proinflammatory cytokines, and reactive oxygen species, all of which may play a role after acute brain injury.

A number of potent p38 inhibitors have been described, the majority belonging to the diaryl imidazole class (e.g. 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole, or SB-203580) [178]. The use of these compounds to study the role of p38 in stroke/neuroprotection in vitro and in vivo has been hampered by low selectivity over related kinases and a lack of demonstrable CNS penetration. Second-generation analogues such as SB-239063 have recently been reported and are claimed to have improved kinase selectivity as well as achieving significant brain penetration. SB-239063 is reported to have an inhibitory concentration IC₅₀ of 40 nM for the p38 α and β isoforms with > 2000 -fold selectivity over other kinases such as p38 γ and δ , ERK, C-Jun N-terminal protein kinase-1 (JNK-1), c-RAF, and PKC α . SB-239063 has been shown to reduce brain injury and neurological deficits in a rat model of cerebral focal ischemia [191–193].

13.7.2.5 Antiadhesion Molecules. Neutrophil migration is mediated by adhesion molecules that are highly expressed on the endothelial surface during stroke and other inflammatory processes. Intercellular adhesion molecule-1 (ICAM-1) is an inducible adhesion molecule that binds to leukocyte integrins and facilitates adhesion and migration. Earlier studies demonstrated that there was a progressive increase in expression of activated microglia, ICAM-1, and invading leukocytes after focal ischemia in rats [194]. Further work revealed that ICAM-1 deficit mice were less susceptible to cerebral ischemia [195].

The direct therapeutic potential of direct ICAM-1 inhibition in ischemic injury has been demonstrated through the use of antisense oligonucleotides against ICAM-1 expression [196] and monoclonal antibodies against ICAM-1-mediated cell adhesion [197]. It was also of interest that the neuroprotective effects were more pronounced in

a transient but not in a permanent focal ischemia model [198], suggesting that reperfusion-associated free radicals and inflammation are key mechanisms involved in this process. However, there was a negative outcome with Enlimomab (anti-human ICAM-1 antibody) in a multicenter acute stroke trial [199].

13.7.2.6 Nitric Oxide Synthase Inhibition (Both Anti-inflammatory and Antioxidant Action). In the last decade several studies have examined the role of nitric oxide (NO) and nitric oxide synthase (NOS) in cerebral ischemia and other cerebrovascular disease. It is well established that a large increase in extracellular glutamate in ischemia can initiate a cascade of calcium-mediated toxicity, lipid peroxidation, and free-radical production. Of particular relevance to the current studies is the fact that glutamate activates the NMDA receptor, which in turn activates NOS, leading to excess production of NO^* . High concentrations of NO^* are toxic and interact with O_2^* to produce the highly toxic peroxynitrite anion (ONOO^-). Therefore, NOS inhibitors have been examined as possible neuroprotective agents [200].

However, many earlier studies gave conflicting results due to multiple isoforms of NOS and lack of selective pharmacological tools. In the mid-1990s the availability of transgenic mice helped to clarify the contribution of nNOS and eNOS in cerebral ischemia and other neurodegenerative diseases. The data generated with the transgenics indicated that there was a reduction in infarct volume after MCAO in mice deficient in nNOS compared with normal mice. In contrast, eNOS knockout mice have enlarged infarcts after MCAO [201].

In parallel with the transgenic work, some newer inhibitors that were more selective for nNOS were reported. The first, 7-nitroindazole (7-NI), inhibited nNOS with an IC_{50} of $0.9 \pm 0.1 \mu\text{M}$ in the rat cerebellum. Initial studies reported that 7-NI inhibits NOS without any effects on blood pressure and is neuroprotective in focal ischemia [202, 203]. Other studies have indicated that 7-NI is beneficial after traumatic brain injury in mice and rats. However, a major drawback with 7-NI is poor solubility, and this has limited the route of administration, which has resulted in suboptimal pharmacokinetics. More recent studies have reported data with nNOS inhibitors such as 1-(2-trifluoromethyl-phenyl) imidazole (TRIM) with better solubility and pharmacokinetics. This compound, which was reported to inhibit mouse cerebellar nNOS and rat lung inducible NOS (iNOS) with IC_{50} values of 28.2 ± 0.5 and $27 \pm 0.9 \mu\text{M}$, respectively, but had an IC_{50} of $1057 \pm 12.2 \mu\text{M}$ on bovine aortic eNOS, was active in models of global and focal ischemia [203, 204]. Another molecule, *N*-[4-(2-[(3-Chlorophenyl)methyl]amino)ethyl)phenyl] -2thiophenecarboximidamide dihydrochloride (ARL 17477), is moderately potent and selective inhibitor of neuronal NOS with IC_{50} of $1.0 \mu\text{M}$ and $17 \mu\text{M}$ on human nNOS and eNOS, respectively. This compound has demonstrated neuroprotective effects in animal models of global and focal cerebral ischemia [205, 206].

Other lines of evidence have suggested that iNOS is an important player in ischemic injury. Earlier studies demonstrated that iNOS is induced during brain injury and that inhibitors such as aminoguanidine could protect against focal ischemia [207, 208]. L-Arginine increases ischemic injury in wild-type but not in iNOS-deficient mice [209]. Aminoguanidine suppresses iNOS activity in mice brain with ischemia to the levels of that in iNOS knockout mice [210]. Inhibitors not based on amino acids which are reportedly selective for iNOS include 1400W [N-(3-aminomethyl) benzylacetamidine] [211] and (1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane

hydrochloride (ONO-1714) [212]. The compound 1400W is a tight-binding inhibitor of iNOS with > 5000-fold selective for human and rat iNOS over eNOS. The compound has been shown to significantly reduce ischemic lesion volume and attenuate neurological dysfunction in a rat model of focal cerebral ischemia [213]. ONO-1714 is a highly potent inhibitor of rat and human iNOS ($K_i = 1.8 \text{ nM}$) with a 10-fold selectivity over eNOS. Neuroprotective activity in vivo has been demonstrated, although the inhibitory activity of ONO-1714 on nNOS is unclear [214].

13.7.3 Antioxidants

There is considerable evidence that oxygen free radicals or reactive oxygen species (superoxide, hydroxyl, NO, etc.) are involved in brain injury after cerebral ischemia and reperfusion injury [215–218]. Agents that prevent free-radical production may therefore prevent ischemic brain damage [217–219]. We have discussed the role of NO in Section 13.7.2, so we will focus on other classes of antioxidants, spin trapping agents, and lipid peroxidation inhibitors in this section.

Recombinant human superoxide dismutase has been reported to attenuate ischemic damage caused by 5 min bilateral carotid artery occlusion in gerbils [220]. Further studies have shown that human Cu–Zn–superoxide dismutase (SOD-1) transgenic mice have a reduced infarct volume after MCAO [221]. It has been reported that copper/zinc–superoxide dismutase prevents the early release of mitochondrial cytochrome *c* in ischemic brain [222] and also prevents extracellular signal-regulated kinase activation [223].

The 21-amino-acid steroids, or “lazaroids,” are potent inhibitors of oxygen-radical-induced and iron-catalyzed lipid peroxidation which have been shown to protect against ischemic damage in several species [218, 219]. It has been demonstrated that 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16- α -methylpregna-1,4,9(11)-triene-3,20-dione, monomethane sulfonate (U-74006F, or Tirilazad), is neuroprotective in global and focal cerebral ischemia [219, 224, 225]. However, other groups have also reported that Tirilazad fails to protect in models of global [226] and focal cerebral ischemia [227]. LY178002 (5-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methylene-4thiazolidinone) has been shown to inhibit both iron-dependent lipid peroxidation and key enzymes of the arachidonic pathway [228]. However, the low aqueous solubility of this compound led to the development of LY231617 (2,6-bis(1,1-dimethylethyl)-4-[[[(1-ethyl)amino]methyl]phenol, hydrochloride) for evaluation in animal models of cerebral ischemia. Oral administration of LY231617 reduced hippocampal CA1 damage in a rat model of 4-VO (4-vessel occlusion) [229], and intraperitoneal administration initiated immediately after reperfusion ameliorated functional and morphological damage induced by global ischemia in rats [228]. Further chemical modification gave rise to LY341122, which was also reported to have neuroprotective actions in both focal ischemia [230] and fluid percussion injury [231] models in rats. Other compounds, such as α -lipoic acid, have been shown to protect against reperfusion injury following cerebral ischemia in rats [232]. A wide range of other natural extracts and products such as green tea and grape seed have been reported in the literature but will not be covered in this chapter.

More recently some nitron structures based on α -phenyl-*N-tert*-butylnitron (PBN) have been investigated [233]. The nitron radical trapping agent disodium 2,4-disulfophenyl-*N-tert*-butylnitron (NXY-059, Cerovive) has been extensively studied

in several models of cerebral ischemia [233–235] and progressed into the clinic. NXY-059 represents a molecule where the preclinical studies included dose–responses, monitoring of plasma concentration, and assessment of therapeutic time window [236]. In addition to studies in focal ischemia the compound also showed efficacy in a rat hemorrhage model [237]. Coadministration of NXY-059 and tenecteplase improved clinical rating scores 6 h following embolic strokes in rabbits [238] and was also evaluated in combination studies with the thrombolytic tissue plasminogen activator in a rabbit small clot embolic stroke model [239]. The compound was also studied extensively in a primate model and provided functional and histological improvement [240, 241]. These studies indicated that NXY-059 was capable of providing functional improvement in primates when treatment was initiated 4 h after the stroke [240], and the functional improvement was also superior to that observed with chlormethiazole and the NMDA antagonist AR-R15896AR [241]. NXY-059 was progressed into clinical development and was well tolerated in patients and achieved concentrations above that required to provide protection in the animal models [243, 244]. The compound is currently under evaluation in two large phase III studies, SAINT I (1500 patients with acute stroke, Europe, Asia, Australia, and South Africa) and SAINT II (1500 patients with acute stroke, North and South America). In addition, there is an ongoing phase II study, CHANT (Cerebral Hemorrhagic and NXY-059 Treatment) with NXY-059. On 4, May 2005, it was announced that a first analysis of data from the SAINT I trial involving more than 1700 patients shows a statistically significant reduction versus placebo on the primary outcome of disability after an acute ischemic stroke ($p=0.038$), as measured by the Modified Rankine Scale (MRS). However, on the National Institutes of Health Stroke Scale (NIHSS), there was no significant difference between the treatment groups in measurement of change in neurological impairment. The clinical significance of these findings need to be assessed in light of the outcome of SAINT II and CHANT. In the same press release the company said the CHANT trial will continue as planned. CHANT is a double-blind, randomized, placebo-controlled, parallel-group, multicenter, phase IIb study to assess the safety and tolerability of Cerovive (NXY-059) in adult patients with acute intracerebral hemorrhage. The independent data safety monitoring board recently conducted a planned safety review on the first 200 patients and recommended the trial to proceed per plan. The trial will involve 150 centers in 21 countries.

13.7.4 Apoptosis and Caspase Inhibitors

Since the late 1990s apoptosis has been proposed as a key mechanism in cell death after cerebral ischemia [244–262]. There were also some reports that apoptosis played only a minor role in global and focal ischemia [253, 254]. Much of the debate was often related to the model of ischemia used and what endpoints were studied and which measures of apoptosis were used.

One of the most common mechanisms of apoptosis is the mitochondrial pathway [245, 246]. In this pathway, ischemia leads to the release of mitochondrial cytochrome *c*, a water-soluble peripheral membrane protein of mitochondria and an essential component of the mitochondrial respiratory chain. This cytochrome *c* is translocated from mitochondria to the cytosolic compartment and interacts with the CED-4 homologue, Apaf-1, and deoxyadenosine triphosphate, leading to activation of caspase-9 [251]. Caspase-9 then activates caspase-3, followed by caspase-2, -6, -8, and -10 activation downstream [251]. Caspase-3 also activates caspase-activated DNase

(CAD), and this cleaves DNA and produces damage and DNA fragmentation that are hallmarks of apoptosis [255–257]. Caspase-11 is also a critical initiator of caspase-1 and -3 activation, and both caspase-11 and caspase-3 knockout (KO) animals have shown reduced apoptosis after focal ischemia. The downstream caspases cleave many substrate proteins, including poly(ADP-ribose) polymerase (PARP) [258]. Substrate cleavage causes DNA injury and subsequently leads cells to apoptotic cell death, but excessive activation of PARP causes depletion of nicotinamide-adenine dinucleotide and ATP, which ultimately leads to cellular energy failure and death. Consistent with these notions, PARP KO mice showed decreased infarct volumes after transient MCAO [258]. In contrast, there are proteins that can prevent caspase activation in the cytosol. The inhibitor-of-apoptosis protein (IAP) family suppresses apoptosis by preventing the activation of procaspases and also by inhibiting the enzymatic activity of active caspases. The second mitochondria-derived activator of caspase (Smac) is also released by apoptotic stimuli and binds IAPs, thereby promoting activation of caspase-3.

The Bcl-2 family proteins have one or more Bcl-2 homology domains and play a crucial role in intracellular apoptotic signal transduction by regulating permeability of the mitochondrial membrane. Some investigators believe that mitochondrial cytochrome *c* is released through the permeability transition pore (PTP) and that Bcl-2 family of proteins directly regulate the PTP. Among these proteins, Bax, Bcl-X_S, Bak, Bid, and Bad are proapoptotic (these proteins are thought to eliminate the mitochondrial membrane potential by affecting the PTP and facilitating the release of cytochrome *c*). Conversely, Bcl-2 and Bcl-X_L function to conserve the membrane potential and block the release of cytochrome *c*. As expected, after focal cerebral ischemia, decreased infarct was observed in Bcl-2 overexpressing transgenic mice [259] whereas Bcl-2 KO mice show an increased infarct.

Receptor-mediated apoptosis can occur when members of the death receptor family, such as the Fas receptor and the TNF receptor, are activated. For example, in the Fas receptor pathway, the extracellular Fas ligand (FasL) first binds to a receptor and an adaptor molecule, Fas-associated death domain (FADD) protein, then activates procaspase-8. Subsequently, caspase-8 activates caspase-3, and this effector caspase cleaves PARP and activates CAD, leading to DNA damage and cell death.

Other pathways such as the mixed-lineage kinases (MLKs) and JNK pathways may also be activated after neuronal stress, inflammation, and ischemia [263]. Thus, the JNK signaling pathway has been reported to mediate Bax activation and subsequent neuronal apoptosis after transient focal cerebral ischemia [264]. A number of recent studies from Serono have demonstrated that the c-Jun NH₂-terminal protein kinase inhibitor, AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl)ethyl]amino]-4 pyrimidinyl)acetonitrile), has neuroprotective properties in global [265] and focal ischemia [266]. Additional studies have reported protective effects with a second JNK inhibitor, SP600125, in a global ischemia model [267].

There is still a lot of debate as to whether antiapoptotic approaches will be suitable therapeutic agents for treating acute brain injury. The type and severity of the ischemic insult in rodents seem to be important. Many investigators have reported evidence for apoptosis in global ischemia model, while necrosis may be more important in situations of focal ischemia. The multitude of apoptotic signaling pathways (which we briefly outlined above) may mean that selectively inhibiting one pathway may not be sufficient to prevent the “apoptotic cascade”. There are concerns that inhibiting endogenous programmed cell death pathways may lead to unwanted side effects.

13.8 SOME SUGGESTED CRITERIA FOR DEVELOPMENT OF NEUROPROTECTIVE DRUG

Based on the failure of many earlier trials and an increased knowledge of both the mechanisms of ischemic injury and the importance of the time window, a number of experts from academia and industry got together in the late 1990s and made a number of recommendations. Many of these issues have been discussed in detail in Section 13.5, but we felt it important to again summarize these (Box 13.2) prior to moving on to approaches to enhance recovery after stroke.

BOX 13.2 CONDITIONS THAT SHOULD BE MET BEFORE A COMPOUND PROGRESSES TO CLINICAL TRIAL

These recommendations are based on the Stroke Therapy Academic Industry Roundtable (STAIR) [268] and suggestions proposed in an article by Green et al. [269]

STAIR Recommendations

- Adequate dose–response studies and serum concentrations measured to define minimally and maximally effective doses.
- Time window studies to confirm efficacy.
- Physiological monitoring should be undertaken.
- Randomized, blinded studies that give reproducible effects (one independent).
- Infarct volume measured and functional tests used, including short- and long-term assessment.
- Small-rodent studies with permanent MCAO; if only model used is transient MCAO, then reperfusion should be targeted in clinic.
- Larger species used for novel, first-in-class compound.
- Studies published in peer-reviewed journals.

Additional Proposals (from [269])

- Histological protection should be >70% in both transient and permanent focal ischemia when drug is given 15–30 min postocclusion. Must show efficacy in models of permanent MCAO.*
- Should provide subcortical and cortical protection.
- Attenuates damage to white matter in brain.
- Time and duration of drug administration should be appropriate to the mechanism of action and the proposed clinical protocol.
- Compound is efficacious as monotherapy.

* The protection afforded should be much larger when compound is started early after occlusion as we feel 70% may be impossible to achieve in certain focal model as there is always a core area that may not be salvageable.

13.9 GROWTH FACTORS, FUNCTIONAL RECOVERY, AND APPROACHES TO REPAIR BRAIN POSTSTROKE

Another opportunity for drug therapy in stroke is that of enhancing functional recovery after the ischemic episode. More than one-quarter of stroke survivors will be left with a residual deficit severe enough to leave them dependent on a caregiver for the “activities of daily life” [270, 271]. The costs of specialized care and physiotherapy are a large economic burden, and patients themselves feel depressed and helpless until they can do the basic day-to-day activities such as eating and dressing. It is well established that there is some spontaneous recovery after stroke [270, 271], and intense physiotherapy in humans [272, 273] or exercise and novel environments in rodents [274–276] can enhance recovery. The ability of the brain to recover (Fig. 13.9) during rehabilitation is encouraging, and in the last 5–10 years a number of investigators have evaluated the ability of growth factors [fibroblast growth factor (FGF-2), brain derived growth factor (BDNF), insulinlike growth factor (IGF-1), glial derived growth factor (GDNF), and bone morphogenic proteins (BMPs)], stimulants (amphetamine), hormones (erythropoietin), neurite outgrowth modulators (anti-NoGo antibodies), and other approaches (bone marrow and stem cells) to improve functional recovery in preclinical models. It is hoped that these pharmacological treatments can augment the spontaneous recovery.

13.9.1 Growth Factors

For many years, there has been extensive research into the use of growth factors to speed recovery and repair the damaged brain [277–279]. In particular, factors such as FGF-2 and BMPs have been evaluated in stroke. FGF-2 has been reported to provide functional improvements in a number of models [280–282]. However, in many cases FGF-2 was administered 30 min–3 h after MCAO and also reduced

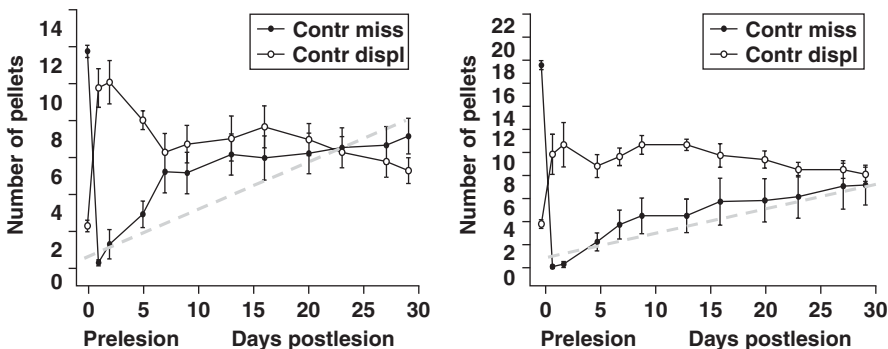


Figure 13.9 Illustrates that there is spontaneous recovery after MCAO in rat. Rats were trained on either a 7 step \times 2 pellet per step (left panel) or a 7 step \times 3 pellet per step (right panel) staircase and then subjected to MCAO. A large deficit in the number of missing (or eaten) pellets was observed one to five days after occlusion. This deficit recovers over time and repeated testing on the apparatus. The recovery is slower when the difficulty of the task is increased by increasing the number of pellets (right panel). Unpublished data from Ann Bond and Michael J. O'Neill. (See color insert.)

infarct volume [280, 281]. Therefore, in some studies it is not clear if the functional recovery was actually due to a decrease in brain damage. However, in some limited studies FGF-2 appeared to improve functional recovery when intercisternal administration was initiated one to three days after MCAO [282]. Additional timecourse studies suggested that FGF-2 could reduce infarct volume when initiated 3 h but not 4 h after MCAO [283].

The use of bone marrow and bone morphogenic proteins has also been attempted [284–288]. Chopp and co-workers have several publications on bone marrow stromal cells (MSCs; an uncharacterized mixed population of plastic-adherent cells) in the treatment of neural injury (see [286] for a review). In a similar way, injection of 1 or 10 μg of human recombinant osteogenic protein-1 (OP-1/BMP-7) into the cisterna magna of rats one and four days after focal cerebral was associated with a marked enhancement of recovery of sensorimotor function of the impaired forelimb and hind limb (contralateral to infarcts) as assessed by limb placing tests [287]. Additional studies suggested that OP-1 (10 μg) given one and three days or three and days, but not seven and nine days after stroke, significantly enhanced recovery of forelimb and hindlimb placing [288]. These improvements are thought to be due to enhanced neurite outgrowth, but enhanced glucose utilization in the basal ganglia ipsilateral to stroke and improved LCBF (local cerebral blood flow) in ipsilateral subthalamus have also been reported [289]. Other studies have suggested that intravenous administration of BMP-7 can also provide functional improvements after focal ischaemia [290].

Another growth factor that has been explored in stroke models is IGF-1 [291–295]. Intraventricular injection of 5–50 μg IGF-1 up to 2 h after occlusion reduced the incidence of infarction and neuronal loss in a dose-dependent manner in all regions ($p < 0.05$), and 50 μg reduced the infarction rate from 87 to 26% ($p < 0.01$). IGF-1 improved outcome compared with equimolar doses of insulin ($p < 0.05$) and did not affect systemic glucose concentrations or cortical temperature [291]. Some studies have reported no effect with IGF-1, but in many cases these studies used lower (2 μg) doses [292]. Topical application of IGF-1 was reported to protect in a rat model of focal ischemia [294].

The role of brain IGFs and IGF binding proteins (IGFBPs) in neuroprotection was further investigated by Neurocrine Biosciences [296]. Investigators used a combination of in vitro and in vivo models of cerebral ischemia and assessed the effects of IGF-I, IGF-II, and high-affinity IGFBP ligand inhibitors (the peptide [Leu24, 59, 60, Ala31]hIGF-I (IGFBP-LI) and the small molecule NBI-31772 [1-(3,4-dihydroxybenzoyl)-3-hydroxycarbonyl-6, 7-dihydroxyisoquinoline]), which pharmacologically displace and elevate endogenous, bioactive IGFs from IGFBPs [296]. Treatment with IGF-I, IGF-II, or IGFBP-LI (2 μmL) significantly ($p < 0.05$) reduced CA1 damage in organotypic hippocampal cultures resulting from 35 min of oxygen and glucose deprivation by 71, 60, and 40%, respectively. In the subtemporal MCAO model of focal ischemia, i.c.v. administration of IGF-I and IGF-II at the time of artery occlusion reduced ischemic brain damage in a dose-dependent manner, with maximum reductions in total infarct size of 37% ($p < 0.01$) and 38% ($p < 0.01$), respectively. In this model of MCAO, i.c.v. administration of NBI-31772 at the time of ischemia onset also dose dependently reduced infarct size, and the highest dose (100 μg) significantly reduced both total (by 40%, $p < 0.01$) and cortical (by 43%, $p < 0.05$) infarct volume. In the intraluminal suture MCAO model, administration of NBI-31772 (50 μg i.c.v.) at the time of artery occlusion reduced both cortical infarct volume (by 40%, $p < 0.01$)

and brain swelling (by 24%, $p < 0.05$), and it was still effective when treatment was delayed up to 3 h after the induction of ischemia [296].

13.9.2 Erythropoietin

Erythropoietin (EPO) produced by the kidney and the liver (in fetuses) stimulates erythropoiesis. In the CNS, neurons express EPO receptor (EPOR) and astrocytes produce EPO [297]. EPO has been shown to protect primary cultured neurons from NMDA receptor-mediated glutamate toxicity. There is now a substantial literature indicating that EPO can protect against global ischemia in gerbils [298], focal ischemia in mice [299] and rats [300], neonatal hypoxia in rats [301], and in a rabbit model of subarachnoid hemorrhage-induced acute cerebral ischemia [302]. It also appears that EPO is a mediator of ischemic tolerance in vitro [303] and in vivo [304] and may therefore be an important endogenous protective factor in the brain. Darbepoetin alfa is a novel erythropoiesis-stimulating protein developed for treating anemia. In animal models, exogenous recombinant human erythropoietin has been reported to be beneficial in treating experimental cerebral ischemia [305].

A recent proof-of-concept clinical trial has been carried out using EPO; 40 patients who had suffered a stroke less than 8 h before trial entry (mean 5.5 h; range 2.9–8.0 h) were randomized into rhEPO or saline treatment groups. A total of 100,000 U of rhEPO was delivered intravenously as equally divided doses over the first 3 days. After 30 days both neurological scores and functionality improved significantly in the active rhEPO treatment group (see [297] for a review). A larger study is in progress to confirm this finding.

13.9.3 Amphetamine and Neurotransmitter Modulators

A number of preclinical studies have suggested that amphetamine (AMP) may promote recovery of function following various types of brain damage in animals. It was found that AMP administered in the hours, days, or weeks after visual cortex lesions could promote recovery of function in the cat [306–309]. Importantly, this recovery appeared to be task specific, with AMP promoting recovery of binocular depth perception but not visual placing reactions [309, 310]. AMP-induced recovery of function has also been observed after sensorimotor cortex lesions in rats [308, 311–313], with these studies suggestive of the fact that AMP can at least accelerate recovery of function, if not promote an absolute recovery. Sensorimotor cortex lesions such as these may offer a particularly relevant animal model of stroke damage for the assessment of postacute pharmacotherapies of stroke [314, 315].

Other small-molecule approaches might be to boost other neurotransmitter pathways. AMPA receptor potentiators enhance glutamatergic transmission and plasticity [316], increase growth factor expression [317, 318], and help enhance functional recovery after nigrostriatal lesion [319, 320]. The utility of AMPA receptor potentiators in post stroke recovery of motor function remains to be established.

13.9.4 Anti-NoGo (IN-1)

Prominent among the several endogenous inhibitors known to limit recovery and plasticity after CNS injury are NoGo (neurite outgrowth inhibitor) and MAG

(myelin-associated glycoprotein) [321]. The effects of these inhibitors on axonal regeneration can be reduced by administration of specific antagonists. Several studies have shown that inhibition of NoGo can enhance spinal cord repair [322, 323]. Additional studies indicated anatomical plasticity in the adult motor cortex after a unilateral sensorimotor cortex (SMC) lesion and treatment with monoclonal antibody (mAb) IN-1 against NoGo, which permits neurite outgrowth from the intact, opposite cortex into deafferented subcortical targets [324]. IN-1 has also been shown to aid functional recovery after MCAO [325, 326] and recently a study indicated that delayed treatment with IN-1 starting one week after stroke results in recovery of function and corticorubral plasticity in adult rats [327].

13.9.5 Hedgehog

Sonic hedgehog (Shh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development. It has been reported that the hedgehog-signaling pathway is present in adult cardiovascular tissues and can be activated *in vivo* [328]. Shh was able to induce robust angiogenesis, characterized by distinct large-diameter vessels. Shh also augmented blood flow recovery and limb salvage following operatively induced hind-limb ischemia in aged mice [328].

The signals that promote regional growth and development of the brain are not well understood. Shh is produced by Purkinje cells of the cerebellum and is a potent inducer of granule cell proliferation. In addition, it has been reported that Shh protein is present in the murine cerebellum during late stages of embryogenesis and is associated with Purkinje cell bodies and their processes [329]. There is also a high expression of the Shh receptor patched in both the adult rat hippocampus and neural progenitor cells isolated from this region [330]. In addition, Shh can elicit a strong, dose-dependent proliferative response in progenitors *in vitro* [330] and is also important in other brain regions [331]. These data suggest that modulation of the shh pathway could help functional repair of the brain after lesion. There have been behavioral improvements following supranigral administration of shh in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated common marmosets [332]. Curis has reported small-molecule modulator hedgehog signaling and identified shh receptor agonists and antagonists [333].

13.9.6 Stem Cells

Pluripotent stem cells are capable of differentiating into any cell type in the body. In recent years there have been extensive research efforts to generate stem cells that can be used to repair the brain [334–336]. ReNeuron has used the conditionally immortal MHP36 line of hippocampal origin (derived from the H-2Kb-tsA58 transgenic mouse) and demonstrated that these cells can repopulate CA1 neurons in models of global ischemia and repair cognitive function [334]. Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field [334]. In addition, delayed grafting of MHP36 cells (two to three weeks after transient intraluminal MCAO) was capable of providing functional improvements in rats [335, 336]. These studies also suggested that these MHP36 cells were able to migrate to areas of damage and to differentiate into neurons and glia.

Other recent research has demonstrated the existence of endogenous stem cells in the adult brain. Attempts to understand the mechanisms of neurogenesis in the intact brain and in response to injury are clearly of interest. Molecular targets that could be modulated with low-molecular-weight drugs (to produce neurogenesis and functional improvement) would be attractive drug targets for the pharmaceutical industry. However, a clear understanding of the biology, about whether it is possible to control neurogenesis and restrict it to particular brain regions, is needed before using these approaches in humans.

13.10 CONCLUSIONS

Stroke is a major cause of death and disability. Over the last 30 years there has been extensive research (i.e., animal models of the disease, the pathways that contribute to cell death, and development of possible pharmacological interventions) to find drugs than can reduce brain injury or enhance functional recovery after stroke. The acute onset and heterogenous nature of stroke make it difficult to find a therapy, and indeed the field has had numerous setbacks in the 1990s. The failures of many stroke trials have resulted in several major pharmaceutical companies reducing or stopping their research efforts in this area. However, much was learned from the early trials and the preclinical criteria and clinical trial design for any new stroke agent are now much better. We anxiously await the second phase III study with Cerovive. It is hoped that the new advances in genomics and proteomics will aid preclinical research and the new imaging modalities should allow better patient selection in the clinic. Continued research is required. There is a huge unmet medical need that must be addressed for patients, their caregivers, and their families.

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14

EPILEPSY: MECHANISMS OF DRUG ACTION AND CLINICAL TREATMENT

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14.1	Introduction	404
14.2	Antiepileptic Drugs and Clinical Epilepsy Types	404
14.3	Clinical Investigation of Epilepsy	405
14.3.1	Prognosis of Epilepsy	407
14.4	Mechanisms of Action of AEDs	408
14.4.1	Modulation of Voltage-Activated Sodium Channels	411
14.4.2	GABA Systems	411
14.4.3	T-Type Calcium Channels	413
14.4.4	Ionotropic Glutamate Receptors	413
14.4.5	Calcium Channel α_2 - δ Subunit	413
14.4.6	Synaptic Vesicle Protein SV2A	414
14.4.7	AEDs with Mixed Actions	414
14.4.7.1	Felbamate	414
14.4.7.2	Topiramate	415
14.5	Relation of AED Mechanism to Clinical Efficacy	418
14.6	Development and Testing of AEDs	418
14.7	Clinical Markers to Predict AED Efficacy	420
14.8	Limitations of Drug Trials for Detecting Idiosyncratic Toxicities	420
14.9	Using Antiepileptic Drugs	421
14.9.1	Generalized Absence Epilepsy	421
14.9.2	Localization-Related Epilepsy	421
14.9.3	Combination Therapy	423
14.9.4	Secondary Generalized Epilepsies	424
14.10	Issues for Special Populations	424
14.11	Drug-Resistant Epilepsy	425
14.12	Alternative Therapies	426
14.12.1	Surgery	426
14.12.2	Electrical Stimulation	427
14.12.3	Diet	427
14.13	Treatment of Acute Seizures and Status Epilepticus	427
14.14	Reflections	428
	References	429

14.1 INTRODUCTION

The epilepsies are a group of chronic neurological conditions characterized by the episodic occurrence of one or more kinds of seizures, which result from the abnormal, synchronous firing of large ensembles of neurons in the brain. Epilepsy is one of the most common neurological disorders, affecting approximately 0.6% of the population in high-income countries [1]. The incidence and prevalence may be higher in lower income regions; the parasitic disease cysticercosis, for example, is a common cause of epilepsy [2]. Patients with uncontrolled epilepsy may suffer severe social, neuropsychological, and economic problems; epilepsy continues to be a stigmatizing disorder, even in industrialized countries [3]. Moreover, patients suffer from increased mortality, due to accidents, suicide, and “sudden unexplained death” [4].

Seizures can be provoked by drugs, such as cocaine, phencyclidine, isoniazid, theophylline, cyclosporin A, and lidocaine; the withdrawal of alcohol, benzodiazepines, or barbiturates; metabolic derangements such as uremia, hypoglycemia, nonketotic hyperglycemia, hyponatremia, and hypocalcemia; and acute head trauma. A person with seizures that occur only in the setting of such transient reversible disorders is not considered to have epilepsy.

The diagnosis of epilepsy is reserved for patients who have had two or more unprovoked seizures. Depending on risk factors such as the presence of a brain lesion or electroencephalographic abnormality, 16–62% of individuals experiencing their first unprovoked seizure will have a second unprovoked seizure within five years [4a]. For many epidemiological studies, patients with a previous diagnosis of epilepsy who have had at least one seizure in the past five years or are taking antiepileptic drugs (AEDs) are considered to have “active epilepsy” [1].

Most clinicians do not treat individuals who have experienced an isolated, unprovoked seizure when there is no evidence of neurological injury, structural abnormality on neuroimaging, abnormality in the electroencephalogram (EEG), or family history of epilepsy. However, individual considerations, including the potential physical, psychological, and vocational consequences of further seizures and of AED therapy, may influence clinical practice.

14.2 ANTIEPILEPTIC DRUGS AND CLINICAL EPILEPSY TYPES

AEDs are the mainstay of epilepsy therapy, although in selected cases surgery is an option. AEDs reduce seizure occurrence but are not known to influence the course of the underlying condition. The efficacy of AEDs is related to both clinical seizure type and epilepsy syndrome classification (based on clinical, electrophysiological, and imaging data). Clinical seizures are broadly classified into partial, beginning with electrical discharges in a limited area of the brain, and generalized, which begin with widespread electrical discharges that involve both sides of the brain at once. Partial seizures are further classified as to whether consciousness (the ability to respond and remember) is impaired (“complex”) or preserved (“simple”). Generalized seizure types include absence, atypical absence, myoclonic, atonic, tonic, clonic, and tonic-clonic. Partial seizures may undergo secondary generalization, in which the localized seizure discharge spreads and generalized tonic-clonic (or more rarely, tonic or

clonic) seizures occur. AEDs may be effective for one clinical seizure type but not another; phenytoin, for example, alleviates partial but not absence seizures.

Although there are a wide variety of syndromes, three broad groups can be discerned: primary generalized epilepsies, secondary generalized epilepsies, and localization-related epilepsies (Table 14.1). Some AEDs, such as ethosuximide in primary generalized absence epilepsy or adrenocorticotrophic hormone (ACTH) in patients with infantile spasms, seem to have syndrome-specific effects.

Childhood-onset absence is the paradigmatic example of primary generalized epilepsy. Affected children may have multiple absence seizures during the course of the day. Absence seizures are characterized by brief (less than 10–20 s) loss of consciousness and, in some cases, eye blinking and slight movement of the mouth or extremities. Absence seizures are associated with 3-Hz spike-and-slow-wave complexes on the EEG and are believed to be caused by abnormal burst firing and oscillatory rhythms in thalamocortical circuits. In general, cognitive and neurological functions are normal, and most patients respond to AED therapy [5].

Patients with secondary generalized epilepsy syndromes, in contrast, may have severe neurological disorders such as tuberous sclerosis, ceroid lipofuscinosis, or Lafora disease. The prognosis is related to the underlying etiology. Even when no specific disorder is present, however, seizures are difficult to treat with AEDs, and additional therapies such as the ketogenic diet may be entertained. Since a clear epileptic focus can be identified only rarely, surgery is an option in a very limited number of cases.

Focal, or localization-related, epilepsies can be due to anything causing focal brain injury, such as trauma, tumors, or encephalitis. However, no underlying cause is apparent in the majority of cases. The most common form of localization-related epilepsy may be temporal lobe epilepsy (TLE). About 50% of patients have a lesion known as mesial temporal sclerosis, characterized by neuronal loss and gliosis in mesial temporal structures (MTSs). In addition to focal or “partial” seizures, patients with localization-related epilepsy may have secondary generalized seizures due to spread of epileptiform discharges. It is interesting that almost all AEDs used in these patients are more effective against the secondarily generalized seizures than the CPSs. Typically, patients with TLE may have several CPSs per week, but no GTCSs for years, as long as they take their AEDs. Focal resective surgery may be very effective for carefully selected patients with TLE [6, 7].

14.3 CLINICAL INVESTIGATION OF EPILEPSY

The patient’s seizure history, family history, and neurological examination can provide important data for epilepsy classification and prognosis. Neuropsychological testing is an important extension of the clinical examination. Deficits in modalities that show functional brain lateralization may help to identify focal epilepsies. Fluctuating test performance may suggest the effects of seizure clusters or AED toxicity, while secular decline can correlate with a worsening epileptic syndrome or underlying progressive etiology [8–10].

The EEG is the most important laboratory test to both detect the presence of a seizure disorder and identify seizure foci in patients with intractable epilepsy being considered for surgery. Early studies suggested that more than 90% of patients will

TABLE 14.1 Broad Syndromic Classification of Epilepsies

Epilepsy Type	Etiology	Clinical Seizure Types	Other Clinical Features	Electroencephalogram	Structural Imaging
Primary generalized epilepsy	Most unknown, presumed genetic; rare ion channel genes implicated	Absence; myoclonus; GTCS	Neurological and neuropsychological exam usually normal	Interictal normal; ictal shows regular generalized spike wave	Usually normal
Secondary generalized epilepsy	Wide range of etiologies, including metabolic disorders, cortical malformations, phakomatoses; many unknown	“Atypical absence” myoclonic, tonic, clonic infantile spasms, GTCS, CPS	Highly variable features related to underlying disease; often developmental, neuropsychological, impairment	Interictal slowing and frequent widespread epileptiform discharges; ictal records often show irregular generalized spike wave discharges	May show wide range of structural and developmental abnormalities
Localization-related (focal)	Focal lesions resulting from developmental defects, trauma, infection, or neoplasms	SPS, CPS, GTCS	May have functional deficits related to seizure focus; usually mild	Focal interictal discharges; ictal discharges begin locally, may generalize	Focal abnormalities may include limited cortical dysplasia, tumors, “mesial temporal sclerosis”

Abbreviations: GTCS, generalized tonic-clonic seizure; CPS, complex partial seizure; SPS, simple partial seizure.

have an abnormal EEG if enough studies are performed [11]. In contrast, less than 3% of patients with no clinical history of seizures had “epileptiform discharges” on initial EEG; 14% of this group later developed epilepsy. Preoperative ictal video-EEG monitoring is used to detect localized seizure onset. In contrast to its diagnostic value, the EEG is used less often to assess the effect of AED therapy [12]. Patients whose clinical seizures are well-controlled can still have interictal EEG discharges, and it has not been shown that trying to suppress these, except in certain restricted instances such as childhood absence epilepsy, provides any therapeutic advantage [13].

AEDs with adverse cognitive effects, such as barbiturates and benzodiazepines, lead to increased β and δ activity on the EEG, but impairment is generally best detected by clinical examination [14].

The main advantage of magnetoencephalography (MEG) is that MEG signal propagation is much less affected by intervening tissue than for EEG so that sources that are not accessible to the EEG may be localized. Coregistration with magnetic resonance imaging (MRI) enhances identification of deep cortical generators of surface-recorded MEG signals. In some cases, MEG may provide better interictal and ictal spike localization and contribute to presurgical evaluation [15].

Neuroimaging has led to a revolution in the treatment of epilepsy and enhanced our understanding of the disease. MRI and positron emission tomography (PET) can detect underlying pathology and focal functional deficits in most patients with localization-related epilepsy. Computed tomography (CT) scanning is much less sensitive and only used to detect central nervous system (CNS) calcifications or other special situations. MRI should be performed in all cases, with the exception of primary generalized childhood absence. PET glucose metabolism and ictal single-photon emission computed tomography (SPECT) blood flow scanning are only performed for presurgical evaluation or research. PET receptor imaging has shown altered benzodiazepine or serotonin receptor binding in patients with focal epilepsies [16, 17]. So far, however, these data have not led to specific pharmacological interventions. MRI has shown evidence for progressive structural changes and FDG–PET progressive metabolic dysfunction in patients with uncontrolled epilepsy, reinforcing the case for aggressive early intervention to prevent neuronal injury from persistent seizures [18, 19].

14.3.1 Prognosis of Epilepsy

It is important to try to predict the prognosis of epilepsy both at the time of first presentation and after initial AED treatment has failed. Several difficulties arise, however. The natural history of untreated epilepsy is unknown, as at least some effective drugs have been available for more than 100 years. Experience in low-income countries, where the majority of patients receive no therapy, suggests that about 30% of patients may experience spontaneous remission or at least a fluctuating clinical course with long seizure-free intervals [20]. Several factors influence recurrence after a single seizure. Patients with symptomatic epilepsy, associated with a brain lesion or injury, have higher recurrence rates than those with no apparent cause or an avoidable precipitant such as transient metabolic derangement [4a]. Recurrence is much higher when a patient has had more than a single seizure: about 75% versus 40% at five years [4a]. Most studies suggest that 60–70% of patients with

“localization-related” epilepsy will become seizure free on AEDs, although the course may fluctuate [21, 22]. Clinical characteristics such as younger onset age may predict an increased risk that seizures become refractory to AED treatment [23]. Patient with primary generalized epilepsy may have a better, and secondary generalized epilepsy a worse, prognosis. Adverse social and psychological effects of epilepsy may persist even if patients eventually become seizure free, particularly when frequent seizures occurred during childhood and adolescence [24].

Randomized studies of early versus delayed treatment of patients who have suffered one or two seizures found a reduction in short-term seizure frequency but no difference in long-term prognosis [25, 26]. However, many neurologists treating patients with epilepsy believe that early intervention and seizure control may help to alleviate the social and neuropsychological consequences of epilepsy, although the risks of AED toxicity have to be considered. There has been increasing recognition that simply suppressing seizures with AEDs may not stop the progression of the underlying epileptic disorder. Conceivably, it may be possible to intervene after a precipitating event such as brain injury to stop the development of epilepsy. While certain treatments can block epileptogenesis in specific animal models of epilepsy (such as the amygdala kindling model), there are as yet no clinical approaches known to retard or check the progression of epilepsy or prevent its occurrence in human subjects [27, 28].

14.4 MECHANISMS OF ACTION OF AEDS

AEDs protect against seizures through interactions with a variety of cellular targets. The actions on these targets can be categorized into four broad groups: (1) modulation of voltage-gated ion channels (mainly sodium and also calcium channels); (2) effects on GABA systems, including enhancement of synaptic inhibition mediated by GABA_A receptors; (3) inhibition of synaptic excitation mediated by ionotropic glutamate receptors; and (4) direct effects on synaptic release machinery [29, 30] (see Table 14.2). The ultimate effects of these interactions are to modify the bursting properties of neurons and to reduce synchronization in localized neuronal ensembles. In addition, AEDs inhibit the spread of abnormal firing to distant sites. Some seizures, including typical generalized absence seizures, are believed to result from thalamocortical synchronization. AEDs effective in these seizure types interfere with the rhythm-generating mechanisms that underlie the synchronized activity in the thalamocortical circuit. (See Table 14.3 and 14.4 for the molecular targets and therapeutic activities of AEDs.)

TABLE 14.2 AED Mechanisms

1.	Modulation of voltage-dependent Na ⁺ or Ca ²⁺ channels (leading to secondary inhibition of synaptic release, particularly of glutamate, or to inhibition of intrinsic bursting)
2.	Enhancement of GABA-mediated inhibition or other effects on GABA systems
3.	Inhibition of synaptic excitation mediated by ionotropic glutamate receptors
4.	Modulation of synaptic release, particularly of glutamate, through direct actions on release machinery

TABLE 14.3 Molecular Targets of AEDs

Drug	Voltage-Activated Sodium Channels	Voltage-Activated Calcium Channels	GABA System	Ionotropic Glutamate Receptors
<i>Predominant Sodium (and Calcium) Channel Activity</i>				
Phenytoin	$I_{\text{NaF}}, I_{\text{NaP}}$			
Carbamazepine	I_{NaF}			
Oxcarbazepine	I_{NaF}			
Lamotrigine	I_{NaF}	HVA		
Zonisamide	I_{NaF}	T type		
<i>Predominant Calcium Channel Activity</i>				
Ethosuximide	? I_{NaP}	T type		
<i>GABA Systems</i>				
Benzodiazepines	—	—	GABA _A R	
Vigabatrin	—	—	GABA-T	
Tiagabine	—	—	GABA transporter	
<i>Mixed</i>				
Felbamate	I_{NaF}	HVA	GABA _A R	NMDA
Topiramate	$I_{\text{NaF}}, I_{\text{NaP}}$	HVA	GABA _A R	KA/AMPA
Phenobarbital	—	HVA	GABA _A R	AMPA
<i>Novel Targets</i>				
Gabapentin	$\alpha_2\delta$ Protein (calcium channel subunit)			
Pregabalin	$\alpha_2\delta$ Protein (calcium channel subunit)			
Levetiracetam	SV2A synaptic vesicle protein			

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ -aminobutyric acid; GABA-T, GABA aminotransferase; GABA_AR, GABA_A receptor; HVA, high-voltage activated; I_{NaF} , fast sodium current; I_{NaP} , persistent sodium current; KA, kainate; NMDA, *N*-methyl-D-aspartate.
Source: Adapted from: [29, 31].

TABLE 14.4 Selected Therapeutic Activities of Marketed AEDs

Antiepileptic Drug	Established and Potential Therapeutic Use
<i>Sodium (and Calcium) Channel Modulation</i>	
Phenytoin	Localization-related epilepsy, GTC seizures <i>Inactive:</i> absence, myoclonus
Carbamazepine	Localization-related epilepsy, GTC seizures <i>Inactive:</i> absence, myoclonus
Oxcarbazepine	Localization-related epilepsy, GTC seizures <i>Inactive:</i> absence, myoclonus
Lamotrigine	Localization-related epilepsy, GTC seizures, absence, myoclonus (JME), Lennox–Gastaut
Zonisamide	Localization-related epilepsy, GTC seizures, myoclonus (JME), absence, infantile spasms, Lennox–Gastaut
Ethosuximide	Primary generalized absence <i>Inactive:</i> localization-related and secondary generalized
<i>Mixed Actions</i>	
Felbamate	Localization-related epilepsy, Lennox–Gastaut syndrome
Topiramate	Localization-related epilepsy, primary GTC seizures, Lennox–Gastaut syndrome, myoclonus (JME)
Phenobarbital	Localization-related epilepsy, GTC seizures <i>Inactive:</i> absence
<i>GABA Systems</i>	
Benzodiazepines	Localization-related epilepsy, GTC seizures, absence, myoclonus
Vigabatrin	Localization-related epilepsy, infantile spasms <i>Inactive:</i> absence, myoclonus
Tiagabine	Localization-related epilepsy <i>Inactive:</i> absence
<i>Novel Targets (Possible Direct Effects on Synaptic Release Mechanisms)</i>	
Levetiracetam	Localization-related epilepsy, myoclonus (JME), absence
Gabapentin	Localization-related epilepsy, GTC <i>Inactive:</i> absence, myoclonus
Pregabalin	Localization-related epilepsy
<i>Obscure Targets</i>	
Valproate	Idiopathic generalized epilepsy (including absence, primary GTC seizures, myoclonic seizures including JME, astatic seizures), localization-related epilepsy, GTC seizures, infantile spasms, neonatal seizures
ACTH	Infantile spasms <i>Inactive:</i> all other seizure types

Note: Therapeutic activities are based on results of controlled trials or open-label trials and general acceptance of utility. In many patients with secondary generalized epilepsies such as the Lennox–Gastaut syndrome, even drugs shown to be effective in controlled trials may only reduce seizure frequency to a limited degree.

Abbreviations: GTC, generalized tonic-clonic seizures; JME, juvenile myoclonic epilepsy. ACTH and glucocorticoids are believed to be effective in the treatment of infantile spasms; the mechanism of action is unknown.

14.4.1 Modulation of Voltage-Activated Sodium Channels

Several AEDs are believed to act largely through modulation of the gating of voltage-activated sodium channels, although effects on other targets including voltage-activated calcium channels may play a role in their seizure protection. These include phenytoin, lamotrigine, carbamazepine, oxcarbazepine, and zonisamide (Table 14.2). Other AEDs that may act, at least in part, through effects on sodium channels include felbamate, topiramate, and valproate. Sodium channel-blocking AEDs inhibit high-frequency repetitive spike firing, which is believed to occur during the spread of seizure activity, without affecting ordinary ongoing neural activity. This accounts for their relatively mild effects on normal brain function. At ordinary hyperpolarized membrane potentials, clinically relevant concentrations of sodium channel-blocking AEDs block sodium channels only weakly. However, upon membrane depolarization, the degree of block markedly increases. Moreover, block accumulates with prolonged or repetitive activation, a property referred to as “use dependence.” Due also to slow onset and recovery of block, normal action potentials, in contrast to the sustained depolarizations of ictal discharges, are relatively unperturbed [32, 32a]. The state-dependent block produced by AEDs that act on voltage-activated sodium channels results from preferential binding of the drugs to inactivated conformations of the channel. These agents act mainly on action potential firing; the drugs do not directly alter excitatory or inhibitory synaptic responses. However, a critical downstream action of AEDs that act on voltage-activated sodium channels may be to reduce action potential–dependent neurotransmitter release, particularly that dependent upon prolonged high-frequency firing as occurs during epileptic discharges [33, 34]. Interestingly, such drugs seem to have a preferential action on glutamate release and only weakly affect GABA release, possibly as a result of differences in excitation–contraction coupling in glutamatergic and GABAergic neurons [35, 36]. Voltage-activated sodium channel block also may reduce the propagation of action potentials from the soma into dendrites and may reduce the dendritic amplification of synaptic potentials [30]. Together, these actions inhibit the spread of epileptiform activity. Blockade of noninactivating sodium currents that arise from alternate gating of the same channels responsible for fast sodium currents and influence initiation and maintenance of epileptiform activity may also contribute to AED effects [37]. Enhanced persistent sodium currents are associated with epilepsy in some forms of the “generalized epilepsy with febrile seizures plus” syndrome [38].

14.4.2 GABA Systems

The GABA system is the second key target for AEDs. Drugs that block GABA_A receptors are well known to induce seizures, and subunit mutations have been associated with inherited epilepsy syndromes [39, 40]. AEDs can interact with GABA systems either through direct effects on postsynaptic GABA receptors or by altering the cellular disposition of GABA. Benzodiazepines are examples of drugs that act on postsynaptic GABA receptors. They are specific for ionotropic GABA_A receptors containing the γ_2 subunit and act to allosterically modulate these receptors to increase chloride channel-opening frequency. This effect enhances synaptic inhibition, resulting in a broad-spectrum anticonvulsant effect. Benzodiazepines can

protect against many seizure types in animal models and in humans, but because of the development of tolerance, they are not generally useful in the chronic treatment of epilepsy. In most epilepsy syndromes, the specific cellular types that are involved in the antiseizure activity of benzodiazepines is not known. However, in the case of absence epilepsy, it is believed that benzodiazepines desynchronize the thalamocortical oscillations underlying generalized spike-wave discharges by specific effects on α_3 -containing GABA_A receptors in the thalamic reticular nucleus [41]. Barbiturates, including phenobarbital, also potentiate GABA_A receptor responses and this is, at least in part, responsible for their antiseizure activity. Presumably because they are not specific for α_3 -containing GABA_A receptors, barbiturates are not active in absence epilepsy and may even aggravate absence seizures. In contrast to benzodiazepines, barbiturates do not appear to increase the frequency of GABA-induced chloride channel opening but instead increase open time probability [42]. In addition to effects on GABA_A receptors, barbiturates modulate other ion channel systems, including calcium and sodium channels, and these actions may contribute to therapeutic activity [43]. Phenobarbital causes sedation and some degree of cognitive impairment at clinically effective doses. Tolerance generally develops to these adverse effects but interestingly not to the anticonvulsant activity [44]. Nevertheless, withdrawal of phenobarbital can lead to seizure exacerbation, as also occurs with benzodiazepines [45].

Drugs that alter the disposition of GABA are vigabatrin, which inhibits GABA metabolism, and tiagabine, which inhibits GABA uptake. Vigabatrin (γ -vinyl GABA) is an irreversible suicide inhibitor of GABA-T, the main metabolic enzyme for GABA, which catalyzes the transfer of an amino group from GABA to pyruvate, forming alanine and succinate semialdehyde [46]. Administration of vigabatrin leads to large elevations in brain GABA levels in animals [47] and humans [48]. In addition, the drug causes a dose-dependent increase in cerebrospinal GABA in human subjects with epilepsy, without affecting the levels of other neurotransmitters, including monoamines [49, 50]. Although GABA-T is present in both neurons and glia [51], the increase in brain GABA levels is predominantly due to inhibition of GABA-T in neurons [52]. While it seems reasonable that these increases in brain GABA would enhance inhibitory tone, in fact, vigabatrin does not potentiate synaptic inhibition [53], and this is consistent with its lack of sedative effects. However, the antiseizure effects of vigabatrin are hard to explain [54]. One possibility is that vigabatrin increases tonic current resulting from the action of ambient GABA on extrasynaptic GABA_A receptors [54–56]. Vigabatrin may cause elevated extracellular GABA levels as a result of efflux of GABA from neurons via reversal of GABA transporters. The enhanced activation of extrasynaptic GABA_A receptors produced by the elevated extracellular GABA could be the critical factor in the anticonvulsant activity of the drug. An alternate hypothesis is that vigabatrin prevents the fading of GABA responses during repetitive activation of inhibitory pathways through reduced function of release-regulating presynaptic GABA_B receptors [57]. Such fading is believed to be an important factor that permits focal epileptiform activity to develop into a full-blown seizure.

In contrast to vigabatrin, the GABA transporter blocker tiagabine [58] does elevate synaptic levels of GABA by inhibiting transport of GABA into nerve terminals and glia, resulting in increased synaptic inhibition [59]. In line with these effects on synaptic inhibition, tiagabine does have sedative side effects.

14.4.3 T-Type Calcium Channels

Low-voltage-activated (T-type) calcium channels play a role in the intrinsic thalamic oscillations that underlie generalized absence seizures [60, 61]. Ethosuximide, which is highly efficacious in the treatment of absence seizures (but not other seizure types) seems to act by inhibition of T-type calcium channels in thalamic neurons [62, 63]. Zonisamide, in addition to effects on voltage-activated sodium channels (Table 14.3), may also block T-type calcium channels [64], thus accounting for its efficacy in absence epilepsy (Table 14.4).

14.4.4 Ionotropic Glutamate Receptors

Ionotropic glutamate receptor-gated cation channels are responsible for most CNS fast excitatory neurotransmission [65]. Selective blockade of NMDA, AMPA, and KA subtypes protects against seizures in animal models [66, 67]. None of the marketed AEDs specifically and uniquely targets ionotropic glutamate receptors. However, several AEDs, including felbamate and topiramate, may, at least in part, act through effects on these receptors (see below).

14.4.5 Calcium Channel α_2 - δ Subunit

Gabapentin and pregabalin are 3-substituted analogs of GABA that were originally synthesized with the intent that they would act on GABA systems. However, neither drug is believed to function through an influence on GABA metabolism or on GABA receptors as do other AEDs that target GABAergic neurotransmission. Rather, both gabapentin and pregabalin are high-affinity ligands for calcium channel α_2 - δ subunits. The α_2 - δ represents a family of four related proteins [molecular weight (MW) \sim 125 kD] encoded by separate genes [68, 69]. Only α_2 - δ_1 and α_2 - δ_2 bind gabapentin and pregabalin with high affinity [70]. The α_2 - δ_1 is expressed ubiquitously in the body whereas α_2 - δ_2 is mainly expressed in the brain and heart. α_2 - δ_1 and α_2 - δ_2 are believed to serve as auxiliary subunits of voltage-activated calcium channels, although it is possible that they have other functions as well. Both proteins form complexes with many calcium channel types (represented by different α_1 isoforms), allosterically enhancing current amplitude and also promoting channel trafficking to the membrane [71]. The mouse mutant *ducky*, which is associated with mutations in the α_2 - δ_2 gene, exhibits spontaneous spike-wave seizures [72]. Similarly, targeted deletion of the α_2 - δ_2 gene results in enhanced seizure susceptibility [73]. These mouse models confirm a role for α_2 - δ_2 in the regulation of seizure susceptibility.

The precise way in which binding of gabapentin and pregabalin to α_2 - δ_1 and α_2 - δ_2 leads to protection against seizures is not fully understood, although there is likely to be an effect on synaptic release of neurotransmitters, including glutamate. Numerous studies have examined the effects of gabapentin or pregabalin on voltage-gated calcium channel function. There are several reports that the drugs reduce calcium current in neuronal cell bodies [74–76]. However, in other studies, gabapentin was inactive [71]. There is, however, agreement that gabapentin and pregabalin reduce calcium influx into presynaptic nerve terminals [77, 78]. This would be expected to reduce neurotransmitter release, and, in fact, there are reports that the release of several neurotransmitters, including glutamate, is reduced by both drugs [79, 79a,

79b]. Recently, several studies have indicated that not only calcium-dependent release of neurotransmitters but also spontaneous, calcium-independent release of individual transmitter vesicles from glutamate synapses is reduced by treatment with gabapentin or pregabalin (see [30]). These results suggest that the actions of α_2 - δ ligands to reduce neurotransmitter release may not require inhibition of calcium influx and therefore may be mediated by an interaction of α_2 - δ (or the calcium channel complex containing α_2 - δ) with synaptic proteins that are involved in the release or trafficking of synaptic vesicles.

14.4.6 Synaptic Vesicle Protein SV2A

The AED levetiracetam is approved for the treatment of localization-related epilepsy and is probably also effective for juvenile myoclonic epilepsy and generalized absence epilepsy (Table 14.4). Levetiracetam has a spectrum of activity in animal models that differs from other agents, and until recently its mechanism of action was obscure [80, 81, 81a]. However, emerging evidence indicates that levetiracetam, like gabapentin and pregabalin, may act through a novel target linked to the synaptic release machinery. In 1995, a saturable and stereoselective specific binding site for [3 H]levetiracetam was described in brain membranes [82]. The binding site was subsequently identified as the ubiquitous synaptic vesicle protein SV2A [83]. Thus, levetiracetam has a novel target that is distinct from that of other AEDs, and it is the first AED that has been demonstrated to bind directly to the synaptic vesicles. SV2A seems to interact with synaptotagmin, which is believed to be the calcium sensor in exocytosis [84]. It is now recognized that SV2A is a member of a small family of homologous proteins that also includes SV2B and SV2C, but only SV2A — the most ubiquitous form — binds levetiracetam. Studies with mice in which the SV2 proteins have been deleted by gene targeting are consistent with a possible role of SV2A in regulating seizure susceptibility, but they have not yet provided insight into the function of SV2A and how levetiracetam binding confers seizure protection. In SV2A knockout mice, brain morphology and indeed the morphology of synapses are normal [85, 86]. However, SV2A knockout mice experience severe seizures. The SV2 proteins do not appear to be required for synaptic transmission or for the uptake or storage of neurotransmitters, although they may play a subtle role in the release process during repetitive synaptic activation (as occurs during seizure activity) by regulating nerve terminal calcium dynamics [86]. Given the way binding to SV2A results in seizure protection, it is likely that there is an influence on synaptic release, which is in accord with the unifying concept that the ultimate action of many AEDs, whatever their molecular targets, is to modulate neurotransmitter release.

14.4.7 AEDs with Mixed Actions

14.4.7.1 Felbamate. Felbamate has a broad spectrum of activity, including efficacy in the treatment of the Lennox–Gastaut syndrome (Table 14.4). Felbamate probably acts through several target interactions, including actions on GABA_A and NMDA receptors. Drugs that block NMDA receptors have powerful anticonvulsant activity in animal models, although it is less clear that such agents are useful in the treatment of human epilepsies [27]. Nevertheless, felbamate is the only AED that targets NMDA receptors at therapeutic concentrations. Structurally, felbamate is an analog

of the sedative–hypnotic drug meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate). Like meprobamate, felbamate potentiates GABA responses via an interaction with a site on the GABA_A receptor that is distinct from the benzodiazepine recognition site [87, 88]. In addition, felbamate blocks NMDA receptor–mediated synaptic responses [89] and inhibits NMDA receptor currents in cultured neurons [87, 90]. On the basis of whole-cell and single-channel recordings, Subramaniam et al. [90] concluded that felbamate acts both by a channel-blocking mechanism and by distinct effects on channel gating. Details of the effects on gating have been defined by Kuo et al. [91], who found that felbamate blocks the late sustained phase of NMDA receptor responses more readily than the initial onset of the response, which may confer selectivity for seizure activity, since prolonged pathological activations would be suppressed more strongly than more rapid, normal NMDA responses. In addition, the block of NMDA receptor responses was greater with high concentrations of NMDA, another factor that would allow the drug to selectively block seizure discharges associated with strong activation of NMDA receptors. Studies with recombinant NMDA receptor subunit combinations have indicated that felbamate selectively blocks NMDA receptors composed of NR2B subunits at lower concentrations than other subunit combinations [92, 93, 93a]. Since NR2B subunits have a restricted distribution in the adult (mainly to the forebrain), this selectivity could contribute to the relatively low neurobehavioral toxicity of felbamate in relation to other NMDA receptor antagonists. Moreover, the subunit selectivity could also account for the clinical utility of felbamate in seizure disorders affecting the immature brain, such as the Lennox–Gastaut syndrome, since NR2B subunits are more abundant in the developing brain.

14.4.7.2 Topiramate. Topiramate is approved for the treatment of localization-related epilepsy, primary GTC seizures, and seizures associated with the Lennox–Gastaut syndrome; it is probably also effective for myoclonic seizures in juvenile myoclonic epilepsy and generalized absence epilepsy (Table 14.4). Several cellular mechanisms have been proposed to underlie the therapeutic activity of topiramate: (1) use-dependent attenuation of voltage-activated sodium currents [94, 95]; (2) inhibition of high-voltage-activated calcium channels [96]; (3) potentiation of GABA_A receptor–mediated currents [97–100]; (4) inhibition of AMPA/KA receptors [101–103]; (5) inhibition of types II and IV carbonic anhydrase isoenzymes [104]; and (6) activation of a steady potassium current [105]. The effects on sodium channels occur at relatively low, therapeutically relevant concentrations and are similar to the effects of other sodium channel-blocking anticonvulsants, particularly phenytoin. In addition to effects on fast sodium currents, topiramate, like phenytoin, blocks persistent sodium currents at low concentrations. Because persistent sodium current may contribute to the initiation and maintenance of epileptiform activity, this action could represent an important factor in the anticonvulsant properties of topiramate. The inhibitory action of topiramate on high-voltage-activated calcium current is of uncertain relevance, since the drug was specific for L-type currents [96] and L-type calcium channel blockers are not effective as anticonvulsants.

Effects of topiramate on GABA_A receptors could contribute to the broad spectrum of activity of topiramate. Topiramate is not active in animal models, such as the pentylenetetrazol test, that are typically sensitive to drugs that positively modulate GABA_A receptors. Nevertheless, the drug does have activity in an absence

epilepsy model and can affect pentylenetetrazol threshold, which is consistent with effects on GABA_A receptors. The activity of topiramate as a modulator of GABA_A receptors varies in different in vitro preparations [101, 106]. Recent evidence indicates that this variability may result from subunit selectivity. In particular, the type of β -subunit type (β_1 versus β_2 or β_3) may strongly influence how topiramate acts on GABA_A receptors.

The activity of topiramate in animal models is compatible with effects on ionotropic glutamate receptors, including NMDA and AMPA/KA receptors. There is no evidence that topiramate blocks NMDA receptors [101]. However, in cultured neurons, it does inhibit responses to KA, an agonist of AMPA and KA receptors [102]. Recently, topiramate was found to be a more potent and efficacious inhibitor of GluR5 KA receptor currents in basolateral amygdala principal neurons than of AMPA receptor currents [106]. AMPA receptors are crucial for excitatory synaptic transmission throughout the CNS, and drugs that substantially block AMPA receptors produce dramatic neurobehavioral impairment [107]. Thus, the finding that topiramate is weak and has low efficacy as an AMPA receptor antagonist corresponds with the clinical observation that the drug is reasonably well tolerated (see Table 14.5 for side effects). KA receptors represent a new potential anticonvulsant drug target [108]. Because of their limited distribution, blockade of GluR5 KA receptors is not expected to be associated with the side effects that would occur with inhibition of AMPA receptors. The inhibitory action of topiramate on GluR5 KA receptors develops slowly, suggesting that it acts indirectly (does not bind directly to the receptor-channel complex). Recently, it has been found that topiramate inhibits phosphorylation of serine 845 of the AMPA receptor GluR1 subunit [109], suggesting that the effect of the drug on AMPA and perhaps KA receptors is due to an alteration in the phosphorylation state of the protein. The relevance of the GluR5 KA receptor blocking activity of topiramate was confirmed in in vivo experiments in mice [110]. In addition to effects on GluR5 KA receptors, topiramate may also affect the more abundant GluR6-containing KA receptors.

The action of topiramate on carbonic anhydrase has been assumed not to contribute to its clinical efficacy because cross-tolerance to the anticonvulsant activity of topiramate does not occur with the classical carbonic anhydrase inhibitor acetazolamide in mice [111]. However, evidence supporting a role for carbonic anhydrase inhibition in the action of topiramate has come from recent studies of GABA_A receptor-mediated depolarizing responses, which can be elicited by high-frequency stimulation of GABA synapses [105]. Such depolarizing GABA responses may promote the generation of seizure discharges. The efflux of intracellular bicarbonate formed by carbonic anhydrase is believed to contribute to depolarizing GABA responses. Topiramate at clinically relevant concentrations strongly inhibited depolarizing GABA responses without affecting hyperpolarizing GABA-mediated inhibitory postsynaptic potentials. This effect is assumed to be due to carbonic anhydrase inhibition as it was mimicked by acetazolamide.

The broad-spectrum anticonvulsant activity of topiramate is likely to result from mixed effects on several target sites, including voltage-activated sodium channels, KA receptors, GABA_A receptor subtypes, and possibly carbonic anhydrase isoenzymes. The effects on ion channels are unlikely to occur through direct modulation of channel gating as is the mode of action of other AEDs that target the ion channel.

TABLE 14.5 Clinical Uses of Antiepileptic Drugs in Treatment of Epilepsy

Drug	Seizure Syndromes	Initial Dose	Titration	Maximum Daily Dose	Neurologic Toxicity	Systemic Toxicity
Phenobarbital	LRE	30 mg	Weekly	4 mg/kg	Sedation, cognitive, behavioral impairment	Hypocalcemia, hypersensitivity
Phenytoin	LRE	300	Loading	600 mg	Cerebellar impairment	Allergic, hematologic, endocrine
Carbamazepine	LRE	100 mg	Weekly	2000 mg	Dizziness, diplopia	Allergic, hematologic, endocrine
Oxcarbazepine	LRE	150 mg	2–4 days	2400 mg	Headache, dizziness	Hyponatremia, rash
Lamotrigine	LRE, SGE, PGE	25 mg	Weekly	1000 mg	Dizziness, diplopia, ataxia	Allergic
Levetiracetam	LRE	5 mg/kg	Weekly	45 mg/kg	Dizziness, psychiatric	Minimal
Topiramate	LRE, SGE	25 mg	Weekly	800 mg	Cognitive, behavioral impairment	Renal stones, glaucoma
Gabapentin	LRE	600 mg	2–4 days	5400 mg	Dizziness, ataxia	Minimal
Zonisamide	LRE, SGE	100 mg	Biweekly	600 mg	Sedation, psychomotor slowing	Renal stones, rare rash
Tiagabine	LRE	4 mg	Weekly	64 mg	Sedation, depression	Low
Valproic acid	LRE, SGE, PGE	10 mg/kg	Weekly	100 mg/kg	Sleepiness, tremor, dizziness	GI, hepatic, endocrine
Ethosuximide	PGE	5 mg/kg	Weekly	40 mg/kg	Sedation, dizziness, behavioral impairment	GI, hypersensitivity
Felbamate	LRE, SGE	15 mg/kg	2–4 days	45 mg/kg	Insomnia, headache	Aplastic anemia, hepatic failure

Abbreviations: LRE, localization-related epilepsy; PGE, primary generalized epilepsy; SGE, secondary generalized epilepsy; GI, gastrointestinal.

Source: Adapted with permission from R. H. Levy, B. S. Meldrum, R. H. Mattson, and E. Perucca, Eds. *Antiepileptic Drugs*, 5th ed. Lippincott Williams & Wilkins, Philadelphia, 2002.

Rather, the pharmacological actions of topiramate seem to be mediated indirectly, possibly through effects on channel phosphorylation.

14.5 RELATION OF AED MECHANISM TO CLINICAL EFFICACY

AEDs that modulate voltage-activated sodium channels, such as phenytoin and carbamazepine, are generally effective in the treatment of localization-related epilepsy and generalized tonic-clonic seizures. However, lamotrigine, another sodium channel modulator, is effective in absence epilepsy and probably other forms of primary generalized epilepsy. The basis for the broader spectrum of activity of lamotrigine is unknown but could relate to actions of the drug on voltage-activated calcium channels [29]. AEDs that act specifically on the GABA system through effects on the disposition of GABA, such as vigabatrin and tiagabine, are generally only effective for localization-related epilepsy. In contrast to vigabatrin and tiagabine, agents that act as positive modulators of GABA_A receptors, such as benzodiazepines, have among the broadest spectrum of efficacy of any AEDs. Thus, the specific way in which an AED acts on its target system in the brain dramatically alters its spectrum of activity. Every drug except ethosuximide, across a wide range of potential mechanisms, provides some benefit for patients with localization-related epilepsy. This is not surprising since the principal registrational clinical trials confirming the efficacy of these agents were restricted to patients with localization-related seizures. Ethosuximide is unique in having a very narrow efficacy range, largely restricted to primary generalized epilepsies and possibly only absence epilepsy. As yet, there is insufficient information on which to derive general principles as to the clinical spectrum of activity of modulators of $\alpha_2\delta$, the target for gabapentin and pregabalin, and SV2A, the target of levetiracetam. However, it is interesting that levetiracetam does appear to have a unique spectrum of activity, with efficacy not only in localization-related epilepsy but also in idiopathic myoclonic seizures (as in the juvenile myoclonic epilepsy syndrome) and probably also primary generalized absence epilepsy. In contrast, gabapentin and pregabalin are probably mainly useful in localization-related epilepsy and generalized tonic-clonic seizures. Drugs that seem to act by mixed or complex mechanisms, such as valproate, felbamate, and topiramate, may have broader clinical spectrums. Valproate is the drug of first choice for the treatment of a wide range of idiopathic generalized epilepsies and secondary generalized epilepsies and is also effective in the treatment of localization-related epilepsy. While its mechanism of action is obscure, it has been proposed that effects on the synthesis and turnover of GABA may be of importance [30]. Both felbamate and topiramate are effective in the treatment of localization-related epilepsy. In addition, felbamate has activity in the Lennox–Gastaut syndrome, one of the catastrophic epilepsies of childhood [112]. Topiramate is probably effective in primary generalized tonic-clonic seizures [113] and in the Lennox–Gastaut syndrome [114].

14.6 DEVELOPMENT AND TESTING OF AEDS

The first effective AEDs, sodium bromide in 1857 and phenobarbital in 1912, were identified because of their sedative properties and subsequently introduced into

epilepsy therapy. Modifications of the barbiturate substrate led in the 1930s to diphenylhydantoin (phenytoin), the first nonsedative AED. Phenytoin was identified as a potential AED through testing in a cat electroshock seizure model. Most subsequent AEDs have been found through screening in a battery of animal seizure models [115]. In a few cases, notably vigabatrin and tiagabine, which were designed to target the GABA system, a rational approach to drug development was used. The Antiepileptic Drug Development Program sponsored by the National Institute of Neurological Disorders and Stroke has aided AED discovery programs in industry and academia [116, 117, 117a].

Clinical development of older AEDs, including phenytoin, ethosuximide, carbamazepine, and valproate, were based on less formal standards than apply today [118]. Beginning in the 1990s, approval of new drugs has been based on large, well-controlled multicenter trials. These trials have demonstrated efficacy mainly in the treatment of localization-related epilepsy. However, it has been noted that such trials do not necessarily demonstrate the overall effectiveness of drugs in clinical practice, which depends on a wide range of factors including ease of use, long-term toxicity, need for clinical monitoring, and cost [119]. Indeed, our knowledge of AED efficacy is related to the peculiarities of their clinical evaluation. Due to the danger of increased seizure frequency in untreated patients with epilepsy, it has been very difficult to devise trial designs in which an experimental drug is compared with placebo. In consequence, most studies in the United States use an “add-on” design in which either experimental drug or placebo is added to stable standard therapy [120–122]. A drug successful in such a trial is approved for use as “adjunctive” therapy.

One strategy to achieve approval for “monotherapy” involves inpatient drug withdrawal (standard during video-EEG monitoring to identify seizure foci in patients being considered for surgery) followed by addition of experimental drug or placebo [123]. This process is expensive, may have relatively higher risks of increased seizures, and does not necessarily mirror conventional clinical treatment due to the effects of baseline AED withdrawal. Dose–response trials have compared high and low doses of the same AED or a low dose of a standard drug (hoping to prevent GTCS but show efficacy of the experimental agent for CPS). However, these approaches, designed to meet regulatory considerations, do not mimic clinical practice and may raise ethical issues [124].

“Outpatient withdrawal to monotherapy” with a drug already approved for adjunctive therapy is another approach that approximates clinical practice in which patients well controlled on two AEDs are often given the option to try to eliminate one of them [125]. Parallel design trials comparing experimental agents with an established drug in patients with new-onset epilepsy have been performed, usually involving agents for which a fair amount of preliminary efficacy data exist [126]. These may suffer from the limitation that equivalence in effect on seizure frequency between “new” and “established” AEDs can be interpreted as evidence for equal effectiveness or equal ineffectiveness.

Most antiepileptic drugs have been tested in adult patients with localization-related, usually temporal lobe, epilepsy. This group makes up the largest number of subjects with uncontrolled epilepsy, and their seizures are relatively easier to control than the atonic or atypical absence seizures of patients with secondary generalized epilepsies. Consequently, there are fewer effective agents for the latter, who suffer as well from more severe neurological disorders. Drugs are mainly tested in adults first

in order to reduce the risk of injury to children, but most patients have epilepsy onset in childhood, and it is important to try to control their seizures as early as possible to prevent adverse social and educational as well as developmental effects.

Unfortunately, then, epilepsy therapy has only a limited “evidence base.” Some investigators have suggested that, given the limitations of the AED development process, “patient preference” may be as reliable a guide in clinical practice as the results of controlled trials [127].

14.7 CLINICAL MARKERS TO PREDICT AED EFFICACY

Ideally, epilepsy treatment should be guided by an “evidence-based” approach, depending on the results of randomized controlled trials (RCTs). Evidence of AED efficacy and clinical decision making should be based on class 1 evidence from controlled, blinded, randomized clinical trials. However, the difficulties and expense of standard clinical AED trials have led to interest in development of surrogate markers of both epilepsy and epileptogenesis. Imaging studies such as PET and MRI are being used to track the effects of pharmacological and surgical treatment in multiple sclerosis and Parkinson’s disease.

None of several potential surrogate markers has been accepted by regulatory agencies or the epilepsy “community” as a reliable indicator of AED efficacy or toxicity. Drug effects on EEG have not been shown to correlate with clinical efficacy, except in patients with primary generalized absence. Transcranial magnetic stimulation (TMS) can be used to evaluate drug effects on cortical excitability. Alterations in TMS measures correlate with AED blood levels, and the specific effects obtained may differ depending upon the AED mechanism of action, but effects on TMS parameters have not yet been shown to reliably reflect seizure frequency reduction [128, 129].

Magnetic resonance spectroscopy has shown that several AEDs increase brain GABA or homocarnosine levels and that the increase may correlate with seizure control [46, 130]. Studies with PET have shown that AEDs associated with cognitive impairment, such as barbiturates, decrease glucose metabolism more than others and that vigabatrin reduces central benzodiazepine receptor binding [131].

Approximately 5% of patients with epilepsy exhibit photosensitivity in which a generalized epileptiform response is observed in the EEG with intermittent photic stimulation (IPS). In these subjects, epileptiform discharges can be reliably evoked in the laboratory at any time by IPS. It has been demonstrated that single acute doses of various AEDs suppress photosensitivity, whereas drugs that do not have antiepileptic actions, even if they produce marked drowsiness, do not [132]. This approach has frequently been used in the early clinical evaluation of potential AEDs to provide an early indication of clinical efficacy and to assess duration of action [133].

14.8 LIMITATIONS OF DRUG TRIALS FOR DETECTING IDIOSYNCRATIC TOXICITIES

Even large-scale randomized controlled clinical trial programs accrue only a few thousand patients at most. Inevitably, rare but serious side effects will not appear until AEDs (or indeed any drugs) come into widespread use. For example, felbamate,

a drug effective for several seizure types that had the advantage of not causing sedation or other manifestations of CNS depression, turned out to cause aplastic anemia at a rate of between 27 and 209 per million users, compared with the general population rate of 2 per million per year [134]. Vigabatrin was found to cause visual field constriction due to retinal toxicity and topiramate acute closed-angle glaucoma [135]. Vigabatrin was never approved in the United States, and its use in Europe has declined. The use of felbamate is restricted to patients that are refractory to other AEDs.

14.9 USING ANTIEPILEPTIC DRUGS

The choice of an AED involves evaluation of its relative advantages, including efficacy, ease of use, and lack of drug interactions, and disadvantages, including toxicity and cost. Table 14.5 shows the range of uses, dosing, and most common or severe side effects for selected AEDs. It is noteworthy that almost all AEDs may cause some mild sedation, to which patients often develop tolerance over time. Table 14.6 provides pharmacokinetic information. Protein binding, half-life, and other parameters shown may be affected by age, drug interactions, pregnancy, and systemic disease. Interactions with other AEDs and therapeutic agents appear to be most extensive for AEDs that induce hepatic enzymes, such as carbamazepine, phenytoin, and phenobarbital. These AEDs can affect the levels of many drug classes, including oral contraceptives, steroids, and anticoagulants. For most AEDs, effective doses are higher on a milligram-per-kilogram basis for children (except newborns) than adults.

AED doses and levels should be taken only as guides, not rigid rules. Recent studies have challenged the value of the routine monitoring of AED plasma levels, particularly in large clinical populations when the newer AEDs are being used [136].

14.9.1 Generalized Absence Epilepsy

In the United States, ethosuximide is the most widely used drug. In Europe, valproic acid has become more popular, particularly in the salt form (divalproex sodium), which has less gastrointestinal toxicity. However, increasing recognition of adverse endocrine effects and the risk of teratogenicity (particularly when doses of more than 1000 mg per day are given) [137], have led to increased use of alternatives such as lamotrigine. The available data do not allow therapeutic distinctions among these three agents [138].

14.9.2 Localization-Related Epilepsy

For patients with localization-related epilepsy, it is reasonable to start therapy with any of several AEDs. Phenobarbital is the most widely used AED worldwide due to its low cost. The long half-life is another advantage, allowing once-daily dosing, which increases compliance. Adverse cognitive effects have reduced use in higher income countries. Phenobarbital is not effective in generalized absence epilepsy and secondary generalized epilepsies, such as the Lennox–Gastaut syndrome. In countries where cost is not an overriding consideration, carbamazepine is probably the most frequently used drug for localization-related epilepsy. Moreover, carbamazepine has become the

TABLE 14.6 Pharmacokinetic Characteristics, Drug Interactions, and Therapeutic Serum Levels of Selected Marketed Antiepileptic Drugs

Drug	Peak Plasma Concentration (h)	Protein Binding (%)	Clearance	$T_{1/2}$ (h)	Drug Interactions	Therapeutic Level ($\mu\text{mol/L}$)
Lamotrigine	1–3	55%	Hepatic	15–60	AEDs	10–60
Gabapentin	2–3 approx. dose	0	Renal	6–7 ^a	Minimal	40–120
Topiramate	1–2	96	CYP3A	5–8	AEDs	^a
Vigabatrin	1–2	0	—	5–7 ^a	—	—
Phenytoin	2–4	15	Mixed	18–23	Lithium, OCs, some AEDs	10–60
Oxcarbazepine	1–2	40	Non-CYP mediated	10–12 (MHD metabolite)	AEDs; oral contraceptives	50–140 (MHD)
Felbamate	2–6	22–25	Hepatic	15–23	AEDs	200–400
Phenobarbital	1–4	40–55	Hepatic	80–130	Extensive	50–130
Phenytoin	2–6	90	Hepatic ^b		Extensive	40–80
Carbamazepine	Slow, variable	70–75	Hepatic	18–55 ^c 12 ^d	Extensive	15–45
Levetiracetam	1–2	0	Renal	6–10	Minimal	—
Zonisamide	3–4	40–60	CYP3A	50–60	Extensive	35–200
Valproic acid	1–2	90 ^e	Hepatic	10–15	AEDs	300–600
Ethosuximide	3–5	0	Hepatic	30–60	AEDs	300–600

Note: MHD, the active 10-monohydroxy metabolite of oxcarbazepine.

Source: Adapted with permission from R. H. Levy, B. S. Meldrum, R. H. Mattson, and E. Perucca, Eds. *Antiepileptic Drugs*, 5th ed. Lippincott Williams & Wilkins, Philadelphia, 2002.

^aUncertain clinical relevance.

^bNonlinear.

^cSingle dose healthy subjects.

^dPatients on chronic therapy.

^eConcentration dependent.

“standard of comparison” in many parallel design AED trials, used most often for patients with new-onset seizures [121, 122]. Carbamazepine induces its own metabolism, so doses may have to be increased after several weeks of therapy, and the drug has to be taken three or four times a day. There are several extended-release forms of carbamazepine available, allowing twice-daily dosing (of the same total amount), which can increase compliance. In a large multicenter, randomized, controlled trial, carbamazepine and phenytoin were superior to phenobarbital and primidone (now rarely used), but due to lower toxicity, not higher efficacy [139]. Phenytoin now is used less frequently than in the past, particularly in Europe, because it commonly causes cosmetic side effects with chronic use (hypertrichosis, gingival hypertrophy, and coarsening of the facial features) and has also been associated with various other chronic toxicities, including cerebellar degeneration, peripheral neuropathy, and folic acid and vitamin D deficiency with osteomalacia.

In the last decade, 10 new AEDs have been approved for marketing. Although comparative data are limited, the parallel treatment comparisons which have been carried out to date have for the most part shown approximate therapeutic equivalence among currently available agents in patients with new-onset localization-related epilepsy [121, 122]. However, in one study carbamazepine seemed to be more efficacious than gabapentin, but at the price of increased side effects. In this parallel group comparison, patients on 600 mg of carbamazepine were more likely to drop out due to toxicity but less likely due to seizures than patients on 900 mg of gabapentin [126]. The overall study completion rate was equal for the two drugs. Even if it is difficult to discern differences in efficacy among the newer agents for the treatment of localization-related epilepsy, each of the new drugs has unique characteristics that may be more important for one patient than another. Such characteristics include propensity to drug interactions, dosing convenience, dose-related side-effect spectrum, and cost. For example, lamotrigine may have less overall toxicity than carbamazepine [140]. However, lamotrigine is associated with a high incidence of skin rashes that rarely may progress to Stevens–Johnson syndrome or toxic epidermal necrolysis. In randomized trials topiramate had more cognitive toxicity than either gabapentin or lamotrigine but also is associated with weight loss and has some efficacy against headache, experienced by many patients with epilepsy [141].

Barbiturates and benzodiazepines both cause sedation and some degree of cognitive impairment at clinically effective doses. Tolerance develops to the latter but not the former during chronic therapy. Although important for treatment of status epilepticus, benzodiazepines have little role in chronic seizure treatment. Phenobarbital, in contrast, due to its low cost, is the most widely used AED in the world and the only agent practical for addressing the enormous treatment gap in low-income countries.

14.9.3 Combination Therapy

If the first AED used is ineffective, clinicians will generally add a second, which can result in improved seizure control. However, only very rarely do combinations of more than two AEDs further increase seizure control [20, 139]. Moreover, patients with localization-related epilepsy, particularly those with mesial temporal sclerosis, are unlikely to respond to any AED if they have failed combination therapy with two

effective drugs. For these patients, alternative approaches such as surgery should be considered. So far, the approach of choosing drugs with two different putative mechanisms of action, although seemingly rational, has not been shown to lead to better seizure control. Nevertheless, there are several important considerations in choosing AED combinations. The potential for drug interactions needs to be taken into account. Several important drugs, such as phenobarbital, phenytoin, and carbamazepine, induce hepatic cytochrome P450 drug-metabolizing enzymes, which can decrease levels not only of other AEDs but also of other medications such as oral contraceptives, anticoagulants, thyroid hormone, as well as vitamin D levels. It is important to give patients taking AEDs calcium and vitamin D supplements. Women particularly are at increased risk for osteoporosis and fractures [142].

It may be helpful to avoid drugs with overlapping side effects, such as two agents that both cause sleepiness, nystagmus, or ataxia. For example, phenytoin and carbamazepine, previously a popular combination, is now used less frequently. The increased rate of side effects reported when patients are taking AED combinations is greater than can be explained by purely additive effects. It may be due to alterations in levels of the original AED or increased production of toxic metabolites.

14.9.4 Secondary Generalized Epilepsies

The treatment of patients with secondary generalized epilepsies such as Lennox–Gastaut remains unsatisfactory. Valproic acid often is the first drug tried. Several others, including felbamate, lamotrigine, and topiramate, have been found to have statistically significant, although limited, efficacy [143a]. The ketogenic diet has been proposed as alternative therapy, but no controlled data on its effects have been reported. For patients with frequent falls and injuries, corpus callosotomy can be considered, although it is a complex procedure with palliative results at best.

Infantile spasms are a particularly severe form of epilepsy, which in about 50% of patients is due to a metabolic brain disorder or tuberous sclerosis [144]. The most common initial therapy in the United States is ACTH, supplemented by valproic acid, lamotrigine, topiramate, and zonisamide. Vigabatrin, not available in the United States, has been reported to be effective for infantile spasms, particularly in patients with tuberous sclerosis, in open-label studies. A recent controlled trial, however, found that vigabatrin was inferior to either ACTH or corticosteroids when seizure outcome was compared at 14 days after starting treatment [145]. Moreover, because it is difficult to monitor the development of retinal toxicity of vigabatrin in infants, treatment with ACTH continues to be the initial approach of choice.

14.10 ISSUES FOR SPECIAL POPULATIONS

Some AEDs may have side effects that affect women more than men. Valproic acid, for example, has been associated with polycystic ovarian syndrome and secondary amenorrhea [146]. The hepatic enzyme inducers can reduce the efficacy of oral contraceptives and lead to unwanted pregnancy; doses may have to be increased. Phenytoin may lead to hirsutism, which is a particular concern for women.

AED teratogenicity often is a concern [147]. All AEDs have teratogenic potential, but reliable risk data are limited. Older drugs such as phenobarbital and phenytoin

have been associated with a variety of fetal malformations, often deriving from studies in which drugs were used in combination. Carbamazepine has been associated with neural tube defects in some studies, although the risk is uncertain. For valproic acid, however, the data show clear increased risk, particularly when doses of greater than 1000 mg per day are used [137]. So far, there are no definite reports of malformations associated with the newer AEDs such as lamotrigine, levetiracetam, or topiramate, but exposures are more limited.

The risk of teratogenicity has to be balanced against the adverse effects of seizures on the fetus. Generally, if a patient who is well controlled on an AED regimen becomes pregnant, AED therapy should be continued and it is not advisable to switch agents. Even in the case of valproic acid, the main period of risk may have passed before the patient realizes she is pregnant. It is important to measure AED levels during pregnancy, as increased fluid volume may lead to decreased plasma levels and loss of control.

Infants usually have decreased AED clearance, but older children need higher drug levels on a milligram-per-kilogram basis. Elderly patients generally have reduced AED clearance and should be given lower drug doses. They are more sensitive to pharmacodynamic effects as well, particularly cognitive toxicity. Some side effects, such as loss of balance, may be more serious in the elderly, leading to falls that can cause fractures. Drug interactions may be a particular concern, since elderly patients may be taking drugs for other medical conditions. In a recent study of patients over 60 with new-onset epilepsy, 150 mg of lamotrigine per day was better tolerated than 600 mg of carbamazepine, with no difference in seizure control [148].

14.11 DRUG-RESISTANT EPILEPSY

It is difficult to predict individual patient responses to AEDs. One of two patients with clinically similar seizures, as well as EEG and imaging findings, may become seizure free forever on a low dose of carbamazepine, for example, whereas the other may remain refractory to maximally tolerated doses of all available AEDs. In addition, some patients may have a good response initially, only to experience a loss of drug effect and development of “intractable” epilepsy. The cause of drug resistance is unknown. In recent years, two hypotheses have been advanced which have received substantial support, at least in preclinical models: the multidrug transporter hypothesis and the drug target hypothesis [149, 150, 150a]. The multidrug transporter hypothesis posits that multidrug efflux transporters overexpressed in epileptic brain regions limit access of AEDs to target sites. In the brain, multidrug transporters, including P-glycoprotein (P-gp), multidrug resistance proteins (MRPs), and breast cancer resistance proteins (BCRPs), are located in the apical (luminal) membrane of endothelial cells that form the blood–brain barrier. These transporters serve to extrude many lipophilic drugs, thus reducing their brain penetration. Several marketed AEDs have been proposed as substrates for P-gp or MRPs, so that overexpression of such transporters at the blood–brain barrier could decrease brain concentrations of these drugs. However, the evidence that all relevant AEDs are substrates is limited [151]; indeed, levetiracetam does not appear to be a substrate [152]. Nevertheless, there is substantial evidence from animal and human studies to support the transporter hypothesis. In particular, numerous studies have shown that

P-gp and other multidrug-resistant proteins are overexpressed in endothelial cells of brain capillaries, and in some instances also in astrocytes and in neurons, in brain tissue resected at epilepsy surgery from patients with drug-refractory epilepsy. In one study, a genetic polymorphism in P-gp, which may enhance transporter activity, was associated with an increased likelihood for pharmacoresistant epilepsy [153, 153a]. However, this association has not been confirmed [154, 155]. The ultimate proof of the transporter hypothesis will require the demonstration that transporter blockers can reverse pharmacoresistance. There are preliminary reports suggesting that this may be feasible, but controlled studies are required [156]. Since multiple different transporters may transport any given drug, highly specific agents may not be effective in all instances.

The drug target hypothesis posits that intrinsic or acquired loss of brain target sensitivity causes AED pharmacoresistance. The target hypothesis is principally based on studies with AEDs on voltage-gated sodium channels. The first in a series of studies examining the hypothesis found that the modulation of sodium current inactivation by carbamazepine in hippocampal CA1 neurons from patients with temporal lobe epilepsy and mesial temporal lobe sclerosis was reduced compared with that in neocortical neurons from the same patients and in CA1 neurons from patients without mesial temporal lobe sclerosis [157, 157a]. In a subsequent study, dentate gyrus granule cell sodium currents in patients with carbamazepine-resistant temporal lobe epilepsy failed to show use-dependent block by carbamazepine [158]. In addition, the fast recovery from inactivation was carbamazepine insensitive. These various results were consistent with the idea that a loss of drug target sensitivity explains the development of drug-resistant epilepsy. A similar loss of sodium channel sensitivity to carbamazepine and phenytoin and in some cases valproate has been found to occur in chronic rat models of epilepsy [157–159]. Alterations of the subunit composition of sodium channels may be present in epileptic animals, but whether this accounts for the pharmacoresistance phenomenon has as-yet not been demonstrated [160].

Little support has been obtained for other proposed mechanisms of pharmacoresistance. For example, a GABA receptor polymorphism associated with intractable temporal lobe epilepsy in an Italian population has not been replicated in France [161, 162]. Moreover, human pathological and imaging studies, including receptor PET and magnetic resonance spectroscopy, have not shown clear differences between drug-refractory and drug-responsive patients.

14.12 ALTERNATIVE THERAPIES

14.12.1 Surgery

Temporal lobectomy for the treatment of temporal lobe epilepsy is the most successful operation. In a randomized study, 58% of surgically treated compared with 8% of medically treated patients were free of disabling seizures at one year [6]. Surgical complications occur in about 1% of patients, and death is extremely rare. Extratemporal resections are less successful; 40–50% of patients may become seizure free [163]. Surgical evaluation includes ictal video-EEG monitoring, a variety of imaging studies, and sometimes intracortical electrode studies, in an attempt to localize epileptic foci. Outcome is closely related to the ease of finding a clearly delineated epileptic focus.

14.12.2 Electrical Stimulation

A variety of electrical stimulation techniques have been proposed for the treatment of epilepsy. Only one, vagal nerve stimulation (VNS), is approved by the Food and Drug Administration (FDA) [165]. VNS requires surgical implantation of a stimulation device with a bipolar lead that is attached to the left vagus nerve. The device, which can be programmed externally, delivers a biphasic current that continuously cycles between on and off periods. Typically, 500- μ s pulses are delivered at 30 Hz for 30 s “on” time and 5 min “off” time. VNS side effects, which are usually mild, include cough, voice alteration, hoarseness, dyspnea, pain, paresthesia, and headaches. Because patients can sense when VNS is active, controlled trials have compared “high” to “low” stimulation parameters. Efficacy was broadly comparable to new AED trials for refractory CPS, with seizure frequency reductions of 25–30% for high versus 6–15% for low stimulation groups. Open-label extension of the trials indicated that the therapeutic response was sustained [165]. Although VNS is a useful adjunctive therapy for patients with localization-related epilepsy not responding to AEDs or with unacceptable toxicity, very few become seizure free. Moreover, since continuing care is needed, VNS does not offer the sense of “cure” attained by patients who become seizure free after resective surgery and can be withdrawn from all AEDs. VNS has only been shown to be effective for complex partial seizures.

Other electrical stimulation approaches include thalamic and substantia nigra stimulation and direct cortical stimulation via implanted devices. Only limited data have been published [164], and any patient considering these approaches should enroll in a formal clinical trial. Transcranial magnetic stimulation provides an alternative means for the activation of cortical circuits that does not require implantation of a stimulation device.

14.12.3 Diet

The high-fat ketogenic diet has been used for about 75 years for the treatment of intractable epilepsy [166]. The mechanism of the diet is unknown but could relate in part to calorie restriction, which seems to be protective by itself [167], and also to increased levels of acetone, which has anticonvulsant properties [168]. Because the diet is unpleasant, restrictive, and easily evaded, it is usually used for young children with severe secondary generalized epilepsies such as the Lennox–Gastaut syndrome. Unfortunately, controlled studies of the diet never have been published, although many physicians treating patients with epilepsy provide anecdotal evidence of efficacy. Although there is general agreement that the diet reduces seizure frequency, there is little information on potential beneficial or adverse long-term effects of the diet on the course of the underlying seizure disorder.

14.13 TREATMENT OF ACUTE SEIZURES AND STATUS EPILEPTICUS

Most seizures, including GTCSs, stop in under 2 min; subsequent postictal confusion may last about 10 min or less [169, 170]. The most important intervention is to prevent physical injury and assure airway patency. Nothing should ever be placed in the mouth of a person during a seizure, as this can lead to severe injury to teeth.

During the postictal period, patients should be gently restrained from wandering or placing themselves in danger.

Status epilepticus has been defined as two or more sequential seizures without full recovery of consciousness between seizures or more than 30 min of continuous seizure activity. However, increasing recognition of the adverse consequences of prolonged seizures has led to a consensus that intervention should begin after 5 min [171]. Generalized tonic-clonic status epilepticus is a medical emergency that requires aggressive treatment, usually first with a benzodiazepine followed by fosphenytoin (a prodrug for phenytoin) or phenytoin, barbiturates, and, in rare occasions, inhalation anesthetics [171, 172]. Intravenous valproic acid is considered first-line treatment for absence status epilepticus and may be a rational choice in myoclonic status epilepticus and in intractable infantile spasms [173]. While barbiturates and inhalational anesthetic agents have traditionally been used to terminate refractory status epilepticus, recent reports suggest that propofol may also be effective and safe [174]. It is important to remember that patients can have brain injury from prolonged seizures even when paralyzed on a respirator in an intensive care unit; EEG monitoring may be needed. The likelihood of persistent impairment following status epilepticus is strongly related to the underlying etiology and also to the duration; the latter can be affected by treatment. Patients may experience hyperthermia, hypoxia, hypotension, and other metabolic derangements that can contribute to a poor outcome.

14.14 REFLECTIONS

The main pharmacological challenge for patients with epilepsy is to provide effective therapy at the lowest possible toxicity. The “new” antiepileptic drugs introduced in the last 10 years or so have provided some improvements, but they have not substantially decreased the proportion of unresponsive patients, particularly those with secondary generalized epilepsy, for whom there are few other options than drug therapy. Moreover, the long-term effectiveness of the new drugs is uncertain. Only 30–50% of patients remain on a new drug after three years [175, 176].

Some investigators have suggested that new approaches to both drug development and clinical testing are needed. The use of a standard battery of animal models for drug screening has been criticized in the belief that the tests used will fail to identify compounds that act in mechanistically new ways. In fact, it is now clear that the “old-fashioned” models often uncover compounds with distinctive profiles of activity in various types of epilepsy and in addition have unexpected efficacy in nonepilepsy conditions, such as neuropathic pain, bipolar disorder, and migraine [177]. Moreover, the animal models provide an opportunity to identify drugs that act in new ways and through new targets, which would not be available if screens against specific molecular targets were used. Nevertheless, there is considerable interest in including models of refractory pharmacoresistant seizures and chronic epilepsy in the panel of tests used in the early identification of anticonvulsant compounds. Perhaps these newer models will identify drugs that are effective in the treatment of patients that fail to respond to currently available drugs.

The greatest roadblock to the development of new AEDs occurs at the stage of clinical testing. The expense and difficulty of recruiting subjects may be particularly

disadvantageous for “niche” compounds that might be effective for an uncommon epilepsy syndrome or a subset of patients. Commercial sponsors have little incentive to conduct trials in these populations. As a consequence, for many of the newer AEDs, studies have not yet been carried out in such niche populations and we do not know whether they might offer special benefits.

Improved treatment of patients with “refractory” epilepsy in the developed world depends on new basic and clinical research. In contrast, for the majority of people with epilepsy in the world who live in low-income countries and receive no treatment at all [178], a great deal can be done with low-cost treatments such as phenobarbital that could potentially lead to seizure freedom in two-thirds of these individuals [179]. Even in the developed world many patients are not receiving the best current care.

A recent National Institutes of Health conference proposed a paradigm shift in our approach to epilepsy, from symptom control to prevention and cure, defined operationally as “no seizures, no side effects” [180]. Cure is a powerful but elusive concept that is as difficult, perhaps, to measure as to achieve. Our understanding of both the basic mechanisms of epilepsy and its clinical course remains limited; seizures may be only one, though dramatic, symptom of a complex and multifaceted illness. Although curing any form of epilepsy will be exceedingly challenging, the accelerating pace of discovery in basic epilepsy research provides the hope that the tools may one day become available to make this goal approachable. It is sobering, though, to realize how few illnesses, apart from acute infectious diseases, can in fact be cured.

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15

PHARMACOTHERAPY FOR TRAUMATIC BRAIN INJURY

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15.1	Neuroprotection	446
15.1.1	Free-Radical Scavengers	447
15.1.2	Anti-Inflammatory Drugs	447
15.1.3	Neurotransmitter Receptor Agonists and Antagonists	449
15.1.4	Neuroactive Steroids and Neurosteroids	450
15.2	Neural Regeneration	452
15.3	Neuroplasticity	453
15.4	Conclusion	454
	References	454

Although stroke, cerebral ischemia, and traumatic brain injury (TBI) share many biochemical, physiological, and morphological processes that lead to the destruction of nervous tissue, the chain of destructive events in stroke is often seen as a distinct disease condition by the medical and scientific community (Fig. 15.1). The distinction may arise in part because there are accepted predictive physiological risk factors for stroke and ischemia and therefore the potential for preventive treatment—diet, physical exercise, and medications to control the levels of circulating cholesterol. In contrast, TBI is often regarded as a random event, caused by external physical forces applied to the head, as when a person is hit by or collides with a high-velocity object as in automobile accidents, falls, contact sports, gunshots, clubbing, and so on. This view of TBI is somewhat misleading, however, because these events are not entirely random. Just as we may choose a diet that increases our risk for vascular disease, we can also choose activities that increase our risk of head injury. This is one reason why males have more than four times as many TBIs as females, so in this case sex can be considered a risk factor for acquired brain damage.

Another distinction between stroke and TBI concerns differences in the age at which these injuries occur. Approximately two-thirds of ischemic stroke cases in this country occur after the age of 60, while TBI typically affects younger adult males involved in high-risk activities (e.g., contact sports, fighting, and driving under the

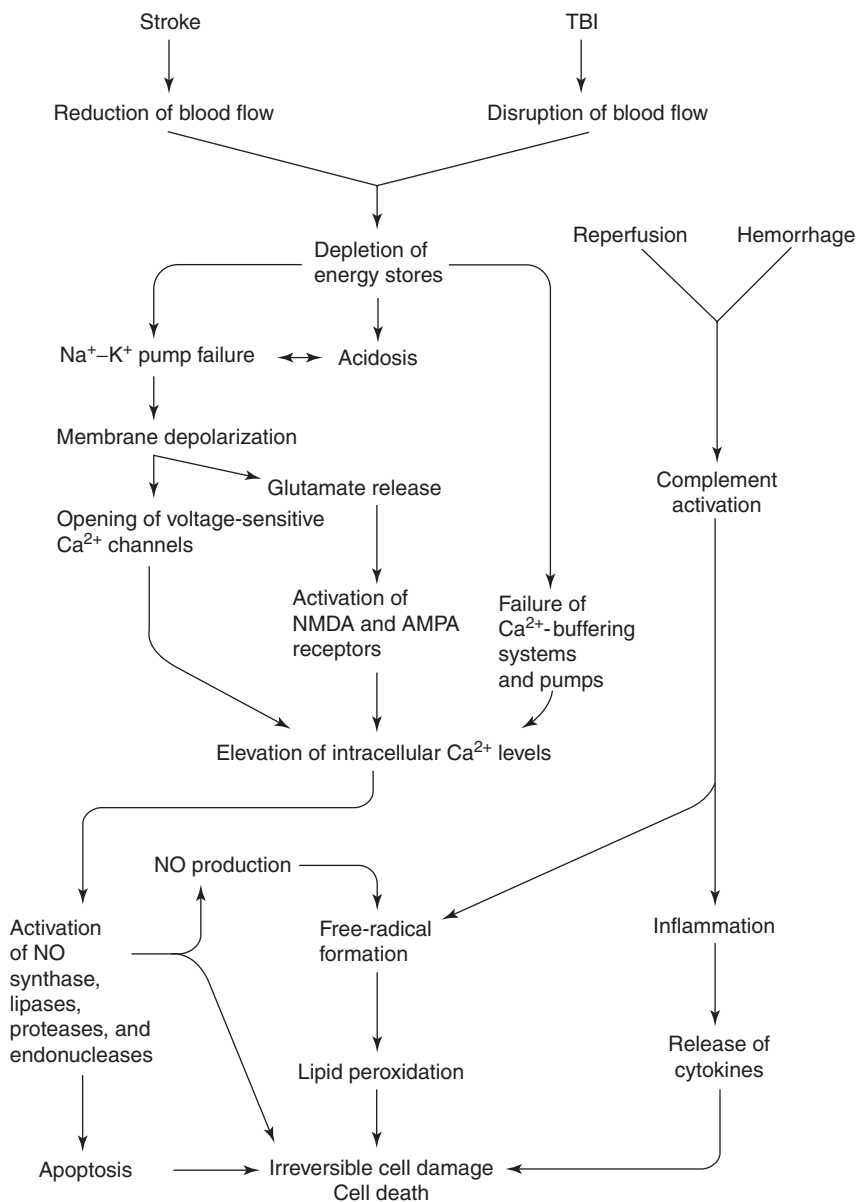


Figure 15.1 Comparison of characteristic event sequences of neurotoxic cascade that follows both ischemic stroke and traumatic brain injury. The cascade has two primary entry points: (1) endogenous sources, such as energy depletion through the lack of oxygen and glucose, and (2) vascular damage from either reperfusion or mechanical injuries. Adapted from [97].

influence of alcohol) and transforms the healthy, active young adult into a person with permanent disabilities. In stroke, the interaction of genes and life-style choices which leads to ischemic attack may take years to develop, so stroke generally affects older adults, although this statistic is changing as the elderly are increasingly subject

TABLE 15.1 Summary of Current Approaches to Treatment of Traumatic Brain Injury

Method to Enhance Recovery	Description	References
Neuroprotection	Administration of compounds that protect neural tissue from cytotoxic and excitotoxic effects of injury cascade	80–87
Regeneration	Administration of trophic factors or transplantation of cells to reestablish normal neural structure	67, 69, 71, 73, 88, 89
Rehabilitation	Using behavioral training or manipulation to stimulate brain to relearn various tasks	78, 79, 90–92
Pharmacotherapy	Administering pharmacological agents to enhance effect of rehabilitation	76, 77, 90, 93–96

to brain injury caused by falls [1]. Whereas stroke is most often caused by blocked arteries, TBI is caused by the transfer of external kinetic energy to the head, causing both impact and shear force injuries to otherwise healthy brain tissue. Because stroke is considered a disease resulting from a variety of long-term pathological conditions, more research is directed to new drug development for this disorder compared to TBI. There is little in the way of preventive treatment for TBI other than improvements in helmets, gun control legislation, active and passive restraint systems in vehicles, and increased enforcement of driving laws. Moreover, there is no equivalent to tissue plasminogen activator (tPA) for TBI and no agreement among researchers about the most effective way to treat both the acute and prolonged cascade of injury events that determine the functional outcome of TBI across the developmental spectrum. Despite the investments in research and development made by large pharmaceutical and biotechnology startup companies to find treatments for TBI, *almost all* clinical trials thus far have failed. (For a discussion of issues in the failures of clinical trials, see [2, 3]).

While neurodegenerative disorders, stroke, and spinal cord injury receive far more attention in the media and far more government funding and research, TBI represents a clinical problem of comparable seriousness. According to the most recent (June 2004) report from the Centers for Disease Control and Prevention (CDC) [1], each year in the United States alone, an estimated 1.4 million people suffer a TBI. Of those, 235,000 are hospitalized and survive—more than 20 times the number of patients with spinal cord injuries. Each year about 50,000 Americans die of a TBI and another 80,000–90,000 suffer a long-term or permanent disability caused by the injury. No age group is spared. In children from birth to 14 years, there are close to 435,000 annual visits to emergency departments associated with TBI. A good number of these incidents occur because of shaken-baby syndrome, and some reports suggest [1] that long-term cognitive disabilities can be the result of this form of TBI. There are also about 300,000 sports-related brain injuries of mild to moderate severity each year in the United States.

TBI victims often report having problems with cognitive functions such as concentration, memory, judgment, emotions, and mood and with sleep and chronic fatigue. Their strength, balance, coordination, and gait can also be affected, so that fine motor skills are disrupted. In more serious and sometimes even in mild cases of TBI, patients lose impulse control and have trouble focusing their attention, maintaining personal relationships, and staying productive in their jobs [4]. The CDC estimates that direct and indirect costs of TBI totaled more than \$56 billion in 1995, so the costs today would be considerably higher.

Because effective acute-stage treatments for TBI are virtually nonexistent, some effort is now being directed to novel pharmacological agents and behavioral therapies that can enhance the rate and extent of central nervous system (CNS) repair. As of this writing, most of the research and development in this area is being conducted by small biotechnology startups as offshoots of university-based neuroscience research. Currently, there is only a handful of National Institutes of Health (NIH)-sponsored clinical trials for TBI and perhaps double that number of privately sponsored clinical trials in the United States. Since most of the nongovernmental research is proprietary, it is not easy to determine exactly what is being done. In general, there are three major types of therapies to increase functional recovery after brain injury (Table 15.1). First is the use of neuroprotective compounds to lessen the impact or “shock” of the acute injury on nervous tissue. A second method is the use of either pharmacological agents or cell transplantation to enhance regenerative mechanisms after injury. Third is the effort to encourage *neuroplasticity* through behavioral and/or pharmacological therapies. The latter, used after cytotoxic events have subsided, may (a) help neurons to produce new synaptic and dendritic processes, (b) stimulate axonal growth cones and the formation of new synapses, (c) stimulate or enhance neurogenesis and reestablish proximal and distal neuronal connections over time, or (d) combine with behavioral therapies to improve and enhance compensatory rehabilitation training.

In the search for new therapies for TBI, it has become clear that the injury itself is not a monolithic or isolated event. Brain injury is a complex cascade of biochemical and morphological processes of varying duration, each of which may contribute to neuronal death and/or repair and regeneration. Until very recently, most head trauma research has focused on what happens to neurons and their transmitters. Now it is becoming clear that repair of the blood–brain barrier (BBB) and the posttraumatic activities of glial cells, macrophages, T cells, and other mediators of inflammation all contribute in critical ways to the prognosis for recovery. Given the growing list of proprietary agents under preclinical investigation and space limitations, we have selected a limited number of representative drugs and categories of drugs that have received more attention as measured by publications in peer-reviewed literature.

15.1 NEUROPROTECTION

In the area of *neuroprotection*, most current drug interventions for brain damage fall into three classes: (a) those given early in the acute stages of the injury process, that is, immediately to not more than a few hours after the TBI, to inhibit inflammation, swelling (edema), cytotoxicity, and apoptosis in vulnerable neural cells; (b) those that agonize or antagonize the synthesis, release, and reuptake of neurotransmitters and neuromodulators which produce a state of overexcitation and oxidative stress,

leading to loss of nervous tissue; and (c) those that are thought to help restore the BBB and cerebral circulation, leading to the rescue of neurons.

15.1.1 Free-Radical Scavengers

Some neuroprotective agents that reduce cytotoxicity work to scavenge damaging molecules such as free radicals. Free radicals react with many sensitive organelles in nervous tissue, including lipids of cellular membranes, DNA, and proteins, changing their conformation and affecting their function. Unfortunately, recent brain injury clinical trials using free-radical scavengers have produced inconsistent results [5]. Despite the disappointing results thus far, agents that reduce lipid peroxidation and tissue nitration are still being sought. There appear to be a number of proprietary free-radical scavenger agents in preclinical evaluation using animal TBI models [6–10]. For example, despite the role of nitric oxide (NO) in the modulation of normal brain functions, some experimental agents are designed to reduce the levels of inflammation-related NO after injury, while other studies report success in neuroprotection by *increasing* vascular-related NO levels after brain trauma to engage its vasodilatory effects [11]. The actions of NO are potentially cytotoxic because its increased levels after brain injury, when it forms along with superoxide into the lipid-soluble, relatively stable free-radical peroxynitrate, can lead to the inhibition of mitochondrial respiration and loss of neurons, glia, and endothelial cells [12–16]. There are also reports that superoxide dismutase, by preventing lipid peroxidation caused by free-radical attack on cell membranes, can prevent neuronal cell loss and reduce cerebral edema in different brain injury models (e.g., [17, 18]). Other agents can trap reactive oxygen species, such as the radical scavenger α -phenyl-*N*-tert-butyl-1 nitron. Among other free-radical scavengers, vitamin E and melatonin have been reported to have beneficial effects in preventing neuronal oxidative stress and eventual cell death [6, 8, 14, 19–22] in animal models of TBI and ischemic injury.

Overall, the successful use of antioxidants and free-radical scavengers to enhance functional recovery in human patients has remained elusive. A number of reasons have been offered for these failures. Some of these agents have difficulty penetrating the BBB, but this fact has limited explanatory power, since the BBB is very often compromised in TBI, and many agents that would not ordinarily pass can find their way into the brain parenchyma. There is much evidence that unless free-radical scavengers are given very early in the injury cascade, they may lose their effectiveness [23, 24]. This is due to the burst of free radicals at the time of the initial injury, disrupting critical cellular functions and generating cytotoxic compounds [24–26]. Because many hours can pass before TBI patients are enrolled in a clinical trial, they cannot be treated in time for the antioxidants to exert their protective effects. In most clinical trials the experimental agent cannot be given until the patient has been transported to hospital and stabilized, informed consent obtained, and so on. In addition, the need to provide oxygen and other medications to prevent life-threatening complications may reduce the effectiveness of the experimental treatments.

15.1.2 Anti-Inflammatory Drugs

Several stages in the inflammatory cascade are amenable to therapeutic intervention. For instance, the modulation of pro- and anti-inflammatory cytokines has been

studied for its effects on the injury cascade. Some chemokines not only promote inflammation and cerebral edema but also act as chemical messengers that activate programmed cell death in neurons, potentiate excitotoxicity, and generate hyperthermia that can exacerbate other stages of the injury cascade. Although some new agents have been shown to reduce proinflammatory cytokines [27, 28], the direct suppression of cytokines or the antagonism of their receptors does not consistently result in improved recovery [29–31].

Another aspect of the inflammatory cascade involves the production of eicosanoids [32, 33]. These paracrine signals are derived from cellular membrane, polyunsaturated fatty acids, such as arachidonate, that have been cleaved off from their polar head group by phospholipases. The free fatty acids can then be processed by specific cellular enzymes and are converted into prostaglandins, leukotrienes, or thromboxanes. There has been recent interest in the inducible form of cyclooxygenase (COX2), the enzyme that initiates the synthesis of prostaglandins [34, 35]. Prostaglandins have been associated with several aspects of the injury cascade, including inflammation, BBB breakdown, and vasoconstriction [36]. Preclinical studies have shown that the administration of COX2 inhibitors can be neuroprotective after TBI, but the effect is not universal [34, 37]. Since current COX2 inhibitors completely block the production of all prostaglandins, treatment with these agents also deprives the injured brain of protective prostaglandins such as prostacyclin, which counteracts many of the vasoconstrictive and proedema signals that occur through other pathways [38, 39].

Corticosteroids, in particular methylprednisolone, have a long history of use in the treatment of TBI. According to some reports, methylprednisolone has been used as treatment for head injury in over 64% of trauma units in the United States [40] and to a much lesser extent (14%) in the British Isles [41] and elsewhere [42]. Over the last decade or so, randomized clinical trials involving several thousand patients were conducted, but the results were considered not altogether consistent [43]. Despite some concerns with the steroid, physicians continued to use methylprednisolone, probably because one trial in over 300 spinal cord injury patients given the drug within eight hours after injury appeared to show beneficial outcomes, with slight improvements in sensory capacity and motor functions six months later [44].

In the late 1990s, because of the increasingly widespread use of methylprednisolone in head injury and because little else was currently available, a very-large-scale, international, randomized, placebo-controlled trial was designed “to confirm or refute” the beneficial effects of the corticosteroid hormones. The aim of the trial (corticosteroid randomization after significant head injury, or CRASH), sponsored by the Medical Research Council of Great Britain, was as follows [45, p. 1321]:

To inform clinical decision-making in an area of increasing global health importance. Reliable demonstration of even a small absolute benefit from corticosteroids would have the potential to avoid thousands of deaths and disabilities. Similarly, because corticosteroids are widely used to treat head injury, reliable refutation of any benefit would protect thousands of patients from possible side-effects and avoid unnecessary cost.

Slightly over 10,000 adults (16 years or older) with TBI and a Glasgow Outcome Score of 14 or less within eight hours of their injuries were randomly assigned to receive either a two-day infusion of methylprednisolone or a placebo. The primary outcome measures were death within two weeks of injury or death or disability within

six months of the trauma. The overall analysis showed, first, that the expected corticosteroid-induced reduction in mortality by two weeks after injury did not occur—there were no differences between the treatment and control groups on this measure. In addition, and against expectation, patients on the steroid showed a highly significant increase in mortality (from an expected 1% decrease in overall mortality to a 2% increase). The statistically significant rise in death rates at two weeks after injury prompted the managers of the study to terminate the trial at enrollment of 10,000 patients rather than the 20,000 originally planned. The six-month outcome measures were not presented, but the many authors of the study conclude that corticosteroids should “*not be used routinely* to treat head injury, whatever the severity” (our italics) [45, p. 1325].

The dramatic failure of the CRASH trial has led to concern about smaller trials, especially those funded by private sources, (see, e.g., [45, 46]). In addition, especially where anti-vivisectionism is strong, questions have been raised as to whether animal studies can usefully inform clinical research at all or provide data to support clinical trials [47]. There may be hidden agendas and other problems associated with studies using small numbers of animals, but the debate, especially in light of the failed CRASH trial, may help to cast doubt on small-scale studies, whether human or laboratory animal. It does highlight the importance of designing very carefully controlled experiments with rigorous attention to statistical power and supported with full statistical expertise [48].

15.1.3 Neurotransmitter Receptor Agonists and Antagonists

Therapies which block the hyperexcitation of the receptors for glutamate have been studied in the laboratory [49]. Like free-radical scavengers, these compounds have not been effective in large phase III clinical trials. In particular, considerable research has focused on agents that can block or antagonize the *N*-methyl-D-aspartate (NMDA) receptor to reduce or modulate excitatory neurotransmission [50]. Since glutamate is a highly excitatory and practically ubiquitous neurotransmitter in the brain, many drugs have been designed to inhibit its actions in order to reduce secondary excitotoxicity. In one recent review of clinical trial data, Willis, Lybrand, and Bellamy [51] reported that there were at that time about a dozen clinical trials using excitatory amino acid inhibitors given within the first 24 h after TBI. Where the data were available for 760 TBI victims in two separate trials, the authors found that “no product has proven to be efficacious for improving the outcomes of brain-injured patients.” The authors of this report did point out, however, that there were issues concerning early termination, proper dosing, and studies being underpowered, all of which could limit the interpretation of efficacy. Other investigators suggest that NMDA receptor antagonists fail because, while glutamate effects are blocked early in injury, over time the neurotransmitter assumes its normal physiological functions. Ikonomidou and Turski [52] speculate that the secondary loss of neurons in stroke and trauma is potentiated by the blockade of glutamate synaptic transmission mediated by the NMDA receptors in the postinjury phase of the disorder. There is some experimental work to support this notion. For example, Hernandez has shown that antiseizure drugs which block neural excitation in rats early in the injury cascade may actually prevent functional recovery by putting vulnerable neurons into a permanent state of diaschisis [53, 54].

Royo et al. [55] suggest that targeting only the NMDA receptors may not be sufficient. For regulating synaptic transmission, strategies that target not only the inhibition of excitotoxicity but also the timing of treatment after injury and the use of multiple agents to affect different aspects of the injury process may be more effective than focusing on the control of one receptor mechanism at a time.

15.1.4 Neuroactive Steroids and Neurosteroids

Several steroids can affect brain function and its response to injury. Among these *neuroactive* steroids, those produced *de novo* in the brain are termed *neurosteroids*. Testosterone and estrogens are neuroactive steroids. (Estrogen can be produced in the brain, but only through the aromatization of testosterone from nonneural sources.) Progesterone and its metabolites, as well as dehydroepiandrosterone and pregnanolone, are classed as neurosteroids because there is substantial evidence that the biosynthetic enzymes required for their synthesis are present in brain tissue.

Secondary brain damage during the acute stages of injury has been successfully reduced in several preclinical models of brain injury using progesterone and estrogen. The benefits of these steroids can be attributed to their actions at several levels of the injury cascade. For instance, both can reduce lipid peroxidation, excitotoxicity, and inflammatory immune reactions due to injury. Progestins and estrogens can be injected systemically or given orally beginning immediately after the onset of injury. In our laboratory investigations, progesterone seems to be particularly effective in reducing cerebral edema and subsequent secondary neuronal degeneration [56, 57]. Thus, as cerebral edema is reduced, neuronal sparing can also be observed in those areas of the brain that have reciprocal connections to the zone of injury. The formation of cerebral edema also appears to be related to the generation of free radicals that initiate lipid peroxidation. One indirect measure of such lipid peroxidation is the extent to which 8-isoprostane, a nonenzymatically produced prostaglandin, is generated following brain damage and acts as a potent constrictor of the cerebral vasculature [58, 59]. Our research shows that postinjury treatment with progesterone can markedly reduce the formation of 8-isoprostane in the first 24 h after contusion of the medial frontal cortex [60]. This change is highly correlated with behavioral recovery, reductions in the size of the necrotic cavity, and enhanced neuronal sparing, which endure long after the treatments have terminated. Our data can be interpreted to suggest that neuronal and behavioral recovery may be due to the scavenging of free radicals and reduction of inflammation in the injury zone. Further support for this notion comes from our recent findings that progesterone can directly inhibit the transcription and activation of a number of inflammatory agents. For example, rats subjected to bilateral contusion injuries of the medial frontal cortex and given just two days of postinjury progesterone treatments showed a substantial reduction of the nuclear factor kappa beta 65 protein and the inflammatory metabolites of complement C3 compared to untreated lesion counterparts.

Inflammatory agents disrupt the BBB, increase immune cell invasion into the damaged brain, and cause cerebral edema and secondary cell loss, so their inhibition can have substantial salutary effects on functional outcome. The treatment effect of progesterone on TBI is extremely robust. Our laboratory has shown that the protective effect of progesterone in controlling cerebral edema can be seen with up to a 24-h delay in administration. Although the literature on the use of estrogens after acute injury is

extensive, much of it involves pretreatment with or simultaneous administration of estrogen. However, recently Yang et al. demonstrated that in an ischemic model of brain injury in the rat estrogen appears to be limited to a 3-h treatment window as determined by 2,3,5-triphenyltetrazolium chloride assay of the necrotic area [61].

γ -Aminobutyric acid (GABA)_A-ergic synapses have also been implicated in the recovery process. GABA is thought to be primarily inhibitory in the cortex, and substances that increase GABA synthesis also appear to block recovery from cerebral injury, especially in the somatosensory system, when administered chronically. Interestingly, progesterone stimulates the GABA_A receptor to reduce excitatory mechanisms, and in this role, it could prevent postinjury epilepsy and excitotoxicity during the early stages after brain injury. This is one example of why developing drug therapies for brain injury is so complex. The problem with managing levels of neurotransmitters as a means of promoting functional recovery is that pharmacological manipulations are extremely sensitive to the elapse of time after the injury. Therefore, increased levels of inhibition that may be needed to block excitotoxicity in the damaged area may actually disrupt recovery if treatment is maintained throughout the course of rehabilitation.

Although many people still think of progesterone as a female sex hormone, its use in males in the acute period after brain injury produces the same beneficial morphological and behavioral consequences as that seen in females [56, 62]. This transgender effect can be attributed to the fact that progesterone is a neurosteroid produced in equal amounts in both male and female brains. It may serve a critical role in maintaining brain function [63]. However, gender difference studies in experimental pharmacology remain virtually nonexistent because investigators do not care to work with animals whose cyclicity would affect outcomes. Therefore, much of the work performed on females of various species is conducted after ovariectomy in a relatively young animal to remove this variable, but the process creates an unnatural state of hormonal deprivation. Such approaches ignore potential drug-hormone interactions that might well be important factors in the outcome of head injury and the prognosis for successful treatment.

We recently completed a National Institutes of Neurological Disorders and Stroke (NINDS) phase I/II clinical trial to test progesterone in 100 patients with moderate to severe TBI. After a year of follow-up, the NIH data safety monitoring board found that there were no serious adverse events attributable to the progesterone administration in any of the patients. These dose and safety parameters have been published [64]. Moreover, 30 days after injury patients in the progesterone group had 57% less mortality than controls. The former group also showed lower intracranial pressure than controls. In the treated patients Glasgow coma scores (GCS) and Galveston Orientation and Amnesia Test (GOAT) scores at 30 days postinjury also revealed that progesterone enhanced functional recovery [64a]. These results must be interpreted with caution because of the relatively small number of patients enrolled in the trial. However, it is promising to note that there were no serious adverse events associated with progesterone administration in either males or females. In addition, the reduction in mortality and better outcome in the treated groups are consistent with a growing number of preclinical studies demonstrating that progesterone is safe and effective in TBI. In fact, thus far this hormone may be the only successful neuroprotective agent with clinical relevance as all other trials have failed to produce positive results or have had too many side effects to risk clinical use.

15.2 NEURAL REGENERATION

Neuroprotective agents act by protecting the brain from secondary injury in the immediate period after the trauma. Recently, research has started to focus on what can be done to stimulate the *regeneration* of neural tissue. Such therapies consist of the *administration of trophic factors*, agents that stimulate the repair, regeneration, elongation, and reconnection of damaged axons or dendrites, and *transplantation of stem cells*, which have the ability to migrate to areas of damage to replace lost neurons and glial cells. But neuronal sprouting is not always beneficial. For example, lesion-induced sprouting of new afferents into the hippocampus can lead to increased seizure activity [65], while some sprouting in the injured spinal cord can produce spasticity [66].

Until only a few years ago, *glial cells* or embryonic stem cells received scant attention as agents that could facilitate recovery from brain damage. Now, glia have been shown to secrete neurotrophic factors that can stimulate sprouting and regeneration, contribute to axonal repair by remyelinating damaged nerve fibers, and directly modify and contribute to neural transmission in response to brain injury [67]. Neuroscientists at Rockefeller University have recently shown that astrocytes in the brains of adult mice can give rise to new neurons in the hippocampus, an area of the brain consistently implicated in memory processing [68]. In the search for better agents to promote functional recovery, it would be advantageous to direct some attention to the effects drugs or stem cell lines might have on the enhancement of the glial response to injury. Such effort would be especially important if glia can be shown to play a role in neurogenesis or regeneration in the adult brain.

A class of proteins and peptides showing promise in head injury treatment are neurotrophic factors produced by the brain itself during development and after injury. Over the past decade, advances in molecular biology have made it possible to produce human trophic factors in genetically engineered bacteria. One problem with their use as therapeutic agents in human patients is that they do not appear to pass through the BBB in sufficient quantity to be clinically effective. However, rats given intracerebral or intraventricular brain-derived trophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF) have shown significant functional and morphological recovery after lesions of the nucleus basalis magnocellularis, the septal nucleus, the hippocampus, and the entorhinal cortex, among others. Notwithstanding good results with laboratory animals, high-quality clinical trial data are still lacking. Despite their potency, delivery of trophic proteins directly into the brain appears to be the limiting factor, because systemic administration by injection appears to have unwanted side effects.

Another exciting new area of research focuses on the use of neural stem cells to repopulate the injured nervous system with tissue that has the capacity to develop into a variety of different organs, including brain cells. The initial experiments with these developing cells have proven quite interesting. In one recent study, Doering and Snyder [69] have shown that when cells from a mouse neural stem cell line were injected into cholinergic basal forebrain areas of mice with fimbria-fornix lesions, these implanted cells differentiated into a neuronal phenotype that expresses choline acetyltransferase and the p75 neurotrophic factor receptor. Chopp and colleagues have injected bone marrow stromal cells into the internal carotid artery after TBI in rats [70–72]. In this study, the injected cells were able to enter the brain parenchyma at the site of injury and differentiate into both neuronal- and astroglial-like

phenotypes. In addition, rats treated with these stromal cells had improved neurological scores compared to their injured controls.

The question of whether the emerging field of stem cell technologies is ready for clinical application has captured the attention of scientists, politicians, ethicists, the public, and the biotechnology corporations—witness the “set-aside” of billions of dollars of public funding for stem cell research by the State of California, with other states (e.g., Massachusetts, New Jersey, Wisconsin) jumping on the bandwagon. Much of the excitement of using stem cells in the treatment of TBI comes from early findings in cell culture research purportedly showing that stem cells taken from different sources in developing embryos could differentiate into any type of cell that might be needed to replace a damaged organ such as liver, heart, kidney, or brain. The rush was on to find and patent stem cells that might be used to treat a variety of devastating disorders such as Parkinson’s disease, amyotrophic lateral sclerosis, Alzheimer’s disease, stroke, and TBI. Although there is no question that further basic research into the effectiveness of stem cells must be done, we think it is premature to transplant them into human patients at this time. There is growing evidence that stem cells implanted into the brain do not act as they do in cell cultures. For example, the literature shows that stem cells retain their progenitor cell characteristics and do not mature into neurons or even specific glial cells. In addition, such cells migrate from where they are placed into the brain and do not necessarily remain in the zone of injury or replace lost or damaged neurons [73]. Although there is a risk of such cells becoming tumorigenic [73a], current thinking is that they may enhance functional recovery by secreting or inducing trophic factors that aid in angiogenesis and/or synaptic and dendritic repair [74]. Until more is known about their mechanisms of action in the brain and since they cannot be removed once placed into patients, the risk of long-term or irremediable negative consequences of such implants calls for delaying clinical testing in humans until such details can be clarified.

15.3 NEUROPLASTICITY

Neuroplasticity therapies can be viewed as attempts to make the brain reorganize. Most research in this area has taken one of two paths. One consists of using drugs to enhance synaptic transmission; the second uses behavioral training techniques to stimulate the brain to restructure. One of the most direct hypotheses about recovery after brain injury is that the *replacement* of essential neurotransmitters lost through stroke or trauma would be beneficial. Just over 50 years ago, medical researchers at Yale University and the University of Moscow administered anticholinesterase agents or cholinergic stimulants to human stroke and head injury patients or to monkeys with unilateral lesions of the motor cortex [75]. More recent experiments have employed amphetamine or amphetamine-like substances to alter norepinephrine levels in brain-damaged animals [76].

In general, amphetamine may affect many different neurotransmitters, so it has been difficult to determine what its specific physiological effects might be. In this sense, it is a nonspecific drug, but it has been repeatedly shown to improve long-term cognitive and motor performance in brain-damaged laboratory animals. One complication of these results is that interaction with environmental stimulation or experience is needed to observe recovery. Thus, brain-injured rats whose movements were restricted during

amphetamine intoxication failed to show any motor recovery compared to conspecifics that were allowed to move freely in their home cages prior to behavioral testing. There is also some limited evidence to suggest that amphetamine given shortly after ischemic stroke will speed the rate of recovery from aphasia [77]. Since some catecholamine antagonists such as haloperidol prevent recovery from brain damage in amphetamine-treated rats, it has been suggested that the restoration of catecholamine levels may be necessary to obtain functional recovery after motor system injuries.

In the last few years, new behavioral therapies have had notable success in stroke victims. For example, some patients lose the use of the arm and hand on the side of the body opposite to the injury. They then use their “good” limb as much as possible to compensate for the lost use of the affected limb. Recently, rehabilitation researchers have demonstrated convincingly that if such patients are forced to use the impaired limb, dramatic and sustained recovery can be observed. The technique is simple: The patient’s good limb is restrained in a sling or tied behind the back, so the only way to accomplish a task is to use the affected hand. When forced to do this, patients show consistent improvement in learning to use the impaired hand [78, 79].

15.4 CONCLUSION

The injury process is a complex cascade of events that can be influenced dramatically by numerous variables, including sex, age, severity and momentum of injury, and hormonal state at the time of trauma, among many others. Therapies may include treatments that do not directly affect neural tissue but instead promote recovery of function by working indirectly on the immune system to reduce inflammation or on the vasculature to increase blood flow and brain metabolism. Because brain injury is a major public health problem, more attention is now being paid to research in this area. Older paradigms which held that the mature brain is incapable of inherent plasticity and repair are being rapidly replaced by a more optimistic vision of CNS plasticity, which will eventually translate into more effective and enduring treatments and therapies for brain damage. One of the major problems in developing treatments for TBI is determining whether effort should be directed to finding a “magic bullet” for the early stage of the injury cascade or to combining agents and the timing of administration to produce the most salutary effects in both the short and long term. Another issue is whether, given the complexity of TBI, small clinical trials will be sufficient to extrapolate to the worldwide population of brain injury victims. Finding funding for even smaller scale studies on TBI that are not based primarily on proprietary agents continues to be difficult. Support for international studies such as the CRASH program is even more unlikely.

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16

DEMENTIA AND PHARMACOTHERAPY: MEMORY DRUGS

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16.1	Diversity of Neural Systems Important for Cognition	461
16.2	Acetylcholinesterase Inhibitors	463
16.2.1	Clinical Experience	463
16.2.2	Considerations from Studies in Nonhuman Primates	463
16.3	Muscarinic Receptor Drugs	465
16.4	Nicotinic Receptor Agonists	467
16.5	Neuroprotective Aspects of Therapy with Nicotinic Receptor Agonists	469
16.6	Glutamate Receptor Ligands	469
16.7	Other Potential Therapeutic Targets	471
	References	474

16.1 DIVERSITY OF NEURAL SYSTEMS IMPORTANT FOR COGNITION

Impaired memory or dementia can occur in conjunction with a number of clinical syndromes, including, head trauma, cerebrovascular accidents, convulsive disorders, nutritional deficits, and drug-associated toxicity. By far the primary disease entity targeted by pharmaceutical research is Alzheimer's disease (AD). AD represents the most common form of dementia among individuals over 65 years of age. It currently affects approximately 4 million Americans and imposes an annual economic burden estimated at between \$80 and \$100 billion. This devastating degenerative condition also inflicts an enormous emotional toll on patients, family members, and caregivers. As the geriatric population inexorably increases, the AD population may increase to epidemic numbers (i.e., in excess of 9 million) by the middle of the twenty-first century.

The gradual loss of memory and other cognitive functions as a consequence of normal aging also is an important concern of older adults. This apprehension has contributed at least in part to the enormous increase in sales of over-the-counter remedies and homeopathic products with claims of memory-enhancing properties.

This demand from a large and ever-increasing elderly population also has provided the basis for a growing interest in pharmacological agents, not just for the treatment of AD but also for the much more common, cognitive decline associated with normal, nondisease aging. A measurable decline in cognitive function that can occur in otherwise healthy aging in humans generally begins after the fifth decade of life. The changes observed in typical (nonpathological) aging are manifested primarily as mild deficits in declarative memory that can result as a consequence of a reduction in the speed of central processing necessary for encoding and retrieval of information [1]. Mild memory deficits that exceed those associated with normal aging but that do not meet the criteria of the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) for a diagnosis of dementia have been referred to as *benign senescent forgetfulness* and *age-associated memory impairment* (AAMI). Beyond these euphemistic references is the diagnosable syndrome mild cognitive impairment, which is thought to be a precursor syndrome to AD [2].

The study of AD, particularly of the neurochemistry of the postmortem AD brain, has provided perhaps a greater level of indirect evidence for important components of cognitive and mnemonic pathways than has the study of the “normal” aging brain. Indeed, the well-known selective vulnerability of basal forebrain acetylcholine-containing neurons in AD has underscored the importance of this neurotransmitter system in memory and perhaps in other behavioral and cognitive functions affected by the disease. Among the degenerative processes occurring in AD, reproducible cholinergic deficits are consistently reported, they appear early in the disease process, and they correlate well with the degree of dementia [3]. Moreover, abnormalities in cholinergic function are frequently reported in other degenerative conditions such as Parkinson’s disease (PD), diffuse Lewy body dementia, and Huntington’s disease. As in AD, such cholinergic deficits often correlate with memory decline and dementia. Extensive AD-related neuropathology also is commonly found in areas normally rich in norepinephrine (locus ceruleus) and serotonin (dorsal raphe nucleus), particularly in the later stages of the disease [4]. The loss of glutamatergic and certain peptidergic pathways in the AD brain also has been reported [4, 5]. These findings have prompted studies designed to reveal the potential for targeting these depleted neurotransmitter systems with respective receptor agonists or other synaptic signal-strengthening drugs. Moreover, disturbances of the cholinergic system in AD may (at least in part) underlie several of the adverse behavioral symptoms (e.g., depression, aggressive behavior, psychosis, and hyperactivity) associated with the disease [6]. These symptoms are often the most troubling to family members and caregivers and often determine the need for institutionalization of AD patients.

The participation of numerous neurotransmitter substances involved in disorders of cognition and in neurodegenerative diseases such as AD is perhaps not too surprising, since memory is represented by several distinct processes and different types of memory are relegated to different (but sometimes overlapping) brain regions. For example, components of the hippocampal formation have been implicated in processing spatial, declarative, and episodic types of memory in humans, primates, and rodents [7–11]. A reasonable argument has been made for the possibility that the hippocampus does not play as important a role in semantic memory [12, 13], with habit learning more dependent upon the striatum [14]. Also, emotional or conditioning learning processes appear to reside within the amygdala [15]. Even within what has been termed working memory or episodic memory, there are separable and

interacting components that may include acquisition (attention), consolidation, and retention [8]. Certain amnesic agents such as scopolamine appear predominantly to affect the acquisition of new learning [16]. Selectivity of action with regard to the components of memory also has been attributed to certain memory-enabling drugs, even within a pharmacological class [17]. Thus, it seems reasonable to conclude that there are several, if not numerous, potential targets for the pharmacological treatment of memory/cognitive disorders. This raises the possibility that drugs that promote activity within different but interacting components of cognitive function may be expected to act additively, if not synergistically, when administered together.

16.2 ACETYLCHOLINESTERASE INHIBITORS

16.2.1 Clinical Experience

Donepezil, a long-acting acetylcholinesterase inhibitor, is widely used in the treatment of mild to moderate AD to improve cognition and memory. Included in this class of agents are the approved drugs rivastigmine and galantamine, though the latter compound also may act as an allosteric activator of central nicotinic acetylcholine receptors. The natural product-derived compound huperzine A is currently in clinical trials. The primary target for these compounds is brain acetylcholinesterase, with the objective of enhancing synaptic levels of the neurotransmitter, leading to improved cognition and memory. This class of drugs is generally considered palliative, and the degree of improvement in cognition in AD is variable and limited in duration. Side effects chiefly reflect the peripheral parasympathomimetic and somatic neuromuscular stimulatory properties of this pharmacological class. The appearance of one or more of these side effects as therapeutic effectiveness is approached can limit the expression of full efficacy for the acetylcholinesterase inhibitors. Also, the continued loss of forebrain cholinergic neurons that occurs in AD likely contributes to the loss of effectiveness. However, recent clinical experience, along with *in vitro* and preclinical studies of neuroprotective potential, suggests that the acetylcholinesterase inhibitors also can delay the progress of the disease by reducing AD-related pathogenesis [18]. Neuroprotective potential has been attributed to galantamine in particular by virtue of its nicotinic actions. Nicotine and many nicotine-like drugs have been demonstrated to enhance cell viability in many assays of neurotoxicity [19].

16.2.2 Considerations from Studies in Nonhuman Primates

Many cognition-enhancing agents, both currently approved medications and those under development, have clinical indications narrowly relegated to AD. The possibility that standard prescriptive drug treatments for AD may also improve cognitive performance in aged nonimpaired or even in young healthy young individuals has not received serious attention. Most animal models utilize subjects with advanced age, pharmacological or surgical interventions, or transgenic manipulation to produce impaired cognitive function. Yet several acetylcholinesterase inhibitors have been shown to improve performance by young unimpaired animals in tasks designed to assess memory capabilities [20–22]. Also, at least one early study

with the short-acting acetylcholinesterase inhibitor physostigmine reported improvement of long-term memory in normal human subjects [23]. This laboratory recently had the opportunity to determine the relevance of age on the ability of donepezil to increase the accuracy of an operant memory task in rhesus monkeys [24]. Subjects ($N=17$) ranged in age from 9 to 29 years old, and they each were well trained to perform a computer-assisted version of the delayed matching-to-sample (DMTS) task. In this paradigm the animals are presented with a colored rectangle on a touch-sensitive screen attached to their cage. Touching within the bounds of the rectangle extinguishes the square and initiates a computer-randomized delay interval. At the end of the delay, two new rectangles are presented of differing colors—one matching the original sample color. A correct response made by touching within the bounds of the correct rectangle is rewarded by the delivery of a flavored food pellet. During a 96-trial session four delay intervals are randomly but evenly presented so that the longer the delay interval, the greater the number of errors made. During sessions in which each animal received donepezil 10 min prior to initiating testing, the effectiveness of the drug (10–100 $\mu\text{g}/\text{kg}$) was so variable as to preclude statistical significance. The response variability, however, could be dramatically reduced (Fig. 16.1a) by the selection of a best dose (greatest degree of improvement in task accuracy). Closer examination of the data revealed that older monkeys generally required higher doses than younger subjects to receive maximal benefit over the dose range tested (Fig. 16.1b). In fact, the regression line fit to the data showing the relationship between best dose and subject age intersected the x axis near zero years of age. This age-dependent difference in donepezil effectiveness could be related to age-related differences in drug metabolism, although it is unlikely that older animals metabolized donepezil faster than younger animals. Also, drug distribution differences should have been minimized with the use of intramuscular administration. One additional ramification of this study is the finding that healthy young subjects received similar benefit from donepezil as did the older group when optimal doses were considered.

The finding that older subjects (even though mildly impaired) required higher doses of donepezil is consistent with the known defects in cholinergic neurotransmission associated with aging [25]. Thus, impaired dynamic functioning of certain brain cholinergic pathways may underlie the symptoms associated with mild cognitive impairment or benign senescent forgetfulness, conditions that may predispose to AD. This might explain why aged monkeys performing versions of delayed response tasks have been of great predictive value in terms of drug efficacy [26, 27].

Additional insight into the mechanism of memory enhancement produced by donepezil treatment could be ascertained from the accuracy–delay duration relationship. That is, after donepezil there was a shift in the slope of this relationship relative to baseline accuracies (Fig. 16.1a). This response characteristic is consistent with an action of donepezil on the retention component of memory [26]. This finding was somewhat surprising considering the general understanding that cholinergic mnemonic pathways play a more dominant role in the attentional components of memory [28]. However, this premise has been based mainly on studies with muscarinic antagonists and other procedures that reduce the output of cholinergic neurons. That acetylcholinesterase inhibitors may more generally increase cognitive processes is consistent with their effectiveness in enabling memory and cognition in nonimpaired individuals. In a recent review of long-term AD treatment with acetylcholinesterase inhibitors, the data revealed improvement in several cognitive

and AD. Part of the appeal is due to the apparent resiliency of specific subtypes of muscarinic receptors relative to the continuing loss of the cholinergic neurons that innervate the postsynaptic cortical and hippocampal cells that express postsynaptic muscarinic receptors [30]. Though M_2 muscarinic receptors have been considered potential therapeutic targets (their inhibition leads to enhanced acetylcholine release), their predominantly presynaptic location links their vulnerability to that of the cholinergic projection neurons upon which they reside. Alternatively, the M_1 subtype exists largely postsynaptically with regard to basal forebrain cholinergic neurons, thus escaping the initial degenerative consequences of AD. This situation offers a parallel to the approach to the treatment of PD. Thus the loss of efficacy to levodopa treatment as PD progresses is compensated to a degree by the use of direct dopamine receptor agonists that can bypass degenerating nigrostriatal dopaminergic neurons. In fact, several positive reports of preclinical efficacy as well as encouraging safety and efficacy data obtained in early clinical trials justified the evaluation of several muscarinic receptor agonists in phase III clinical trials. However, the M_1 -preferring compounds cevimeline, milameline, sabcomeline, Lu25-109, [5-(2-ethyl-2H-tetrazol-5-yl)-1,2,3,6-tetrahydro-1-methylpyridine], and talsaclidine have been associated with poor efficacy, bioavailability problems, or side effects [31, 32] thereby reducing enthusiasm for this pharmacological class. Improved M_1 selectivity, however, could result in more useful muscarinic receptor agonists for the treatment of cognitive disorders. Most of the dose-limiting side effects are gastrointestinal because of overlapping activity at peripheral M_3 muscarinic receptors. Studies with the M_1 -preferring agonists talsaclidine and WAY-132983 [(3*R*,4*R*)-(–)-3-(3-hexyl-sulfanylpiazin-2-yloxy)-1-azabicyclo[2.2.1]heptane hydrochloride] in monkeys revealed significant improvements in DMTS accuracy to each drug; however, in both cases, significant gastrointestinal side effects were evident at the most efficacious doses [33, 34].

The synthetic approach developed by Fisher and his colleagues [30] has provided a new series of muscarinic receptor agonists that exhibit high potency and selectivity for the M_1 subtype. One of these, AF102B (cevimeline, Evoxac), is approved for use in the treatment of Sjogren's syndrome. It is expected that AF102B or one of the other compounds in this series ultimately will be approved for the treatment of AD. The importance of this approach not only resides in the ability of this pharmacological class to improve cognition and memory, but also M_1 receptor agonists have been shown to (1) stimulate non-amyloidogenic α -secretase-processing of the amyloid β precursor protein (APP), (2) inhibit the β -secretase processing pathway, and (3) decrease the levels of phosphorylated tau [35]. Should these properties translate to the clinical situation, in AD, M_1 agonist treatment would prove more than just palliative.

Despite the limitations mentioned above regarding the vulnerability of the M_2 muscarinic receptor in AD, this subtype also should continue to be explored as a therapeutic target. Particularly early in the disease process prior to the loss of significant populations of forebrain cholinergic neurons, inhibition of the M_2 subtype has the potential to increase the release of acetylcholine, and drugs in this class have been demonstrated to increase performance in various memory-related tasks [36]. Since cardiac muscarinic receptors are largely M_2 , heart and vascular side effects could limit the use of this pharmacological class. One approach might be the development of compounds with M_4 -selective antagonism, since this subtype func-

tions like M_2 in the CNS but the receptors are not significantly expressed in the heart [37, 38].

16.4 NICOTINIC RECEPTOR AGONISTS

Nicotinic acetylcholine receptors consist of pentameric transmembrane complexes of distinct subunits termed α , β , γ , δ , and ϵ . In brain, only α and β subunits exist, though there are eight unique α subunits and four β subunits. Subunits α_1 , α_2 , α_3 , α_4 , α_5 , and α_6 , and subunits β_1 , β_2 , β_3 , and β_4 can form hetero-oligomeric nicotinic receptors, whereas α_7 , α_8 , and α_9 subunits can form homo-oligomeric nicotinic receptors. The α subunit contains the ligand binding domain. Many of the functional subunit combinations have been identified in artificial systems [39]. However, it is not yet clear which specific subtypes upon activation would provide the best therapeutic/side-effect profile. For the heteromeric receptor subtypes it has been difficult to develop high subtype selectivity. This could be due to the finding that the ligand selectivity is largely conferred by the β subunit and there are only two, β_2 and β_4 , that appear to have physiological significance. Another feature of nicotinic pharmacology is the ability of the receptor to rapidly desensitize, limiting the action of an agonist. It has been suggested that in certain instances it is nicotine-induced desensitization that produces the desired physiological response. The desensitization of nicotinic receptors usually occurs at concentrations of nicotine much lower than those required for inducing an agonist-like response (e.g., neurotransmitter release). These lower desensitizing concentrations of nicotine are more commensurate with the *in vivo* doses required for cognition enhancement. One possibility is that desensitization disinhibits the tonic cholinergic modulation of a secondary, possibly γ -aminobutyric acid (GABA)-ergic, inhibitory interneuron.

Despite the preponderance of muscarinic cholinergic drugs that are used in various clinical situations relative to nicotinic drugs, the development of new nicotinic receptor agonists, for the treatment of cognitive disorders has greatly outpaced that for muscarinic drugs. The last decade saw the first of a series of novel nicotinic receptor agonists, including ABT-418 [(*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole], SIB-1553A [(\pm)-4-{[2-(1-methyl-2-pyrrolidinyl)ethyl]thio}phenol hydrochloride], and RJR-2429 [(6)-2-(3-pyridinyl)-1-azabicyclo[2.2.2]octane], each of which was shown to exhibit some favorable subtype selectivity for the $\alpha_4\beta_2$ receptor over the α_7 receptor. This laboratory participated in studies with ABT-418, evaluating it in versions of our DMTS task in monkeys. The results confirmed that the compound did enhance accuracy in tests of cognitive performance with similar potency and effectiveness to nicotine [40, 41]. Notably impressive was the ability of the drug to reverse the decrease in accuracy caused by a task-relevant distractor—our primate model for attention-deficit disorder [42]. Clinical studies with ABT-418 demonstrated its effectiveness in individuals with AD, PD, and attention, deficit disorders [43–45].

ABT-418, SIB-1553A, and RJR-2429 provided an important proof of concept as they each exhibited a reduced profile of serious side effects relative to nicotine. The nicotine-induced side effects, including cardiovascular alterations, gastrointestinal distress, and perhaps most importantly abuse liability, were all reduced relative to nicotine in these early compounds. However, nicotine and many of the drugs that

target high-affinity nicotinic receptors, primarily the $\alpha_4\beta_2$ subtype, exhibit a rather narrow therapeutic window, often less than one log unit. The narrow therapeutic window amplifies the issue of side effects, and it still remains to be determined whether a synthetic nicotinic receptor agonist will prove safe and effective in clinical trials. The clinical therapeutic potential for synthetic nicotinic drugs might first be realized not through studies in individuals with cognitive disorders but through the treatment of tobacco dependence. Pfizer's new antismoking therapy varenicline [6,10-methano-6*H*-pyrazino[2,3-*h*][3]benzazepine, 7,8,9,10-tetrahydro-, (2*R*,3*R*)-2,3-dihydroxybutanedioate (1:1)] currently is being studied in phase III clinical trials. Varenicline is a partial agonist that is specific to the $\alpha_4\beta_2$ nicotinic receptor subtype. The concept is that partial agonism would reduce nicotine craving without itself being rewarding or addictive. Varenicline's introduction to the world market would solidify the proof of concept for a "safe" and nonaddictive nicotine-like compound, and it will lend credence to the use of synthetic nicotinic receptor agonists for other indications.

In addition to reducing the expression of nicotinic side effects, synthetic nicotinic receptor agonists have the potential to be targeted to different receptor subtypes that have relevance for one disease over another. For example, SIB-1553A was reported to exhibit some selectivity for subtypes possessing β_4 subunits (e.g., $\alpha_2\beta_4$), whereas SIB-1508Y [(*S*)-(–)-5-ethynyl-3-(1-methyl-2-pyrrolidinyl)-pyridine] exhibited selectivity for $\alpha_4\beta_2$ receptors [46]. These differences in receptor subunit preference resulted in differing profiles of neurotransmitter-releasing activity. Though both compounds (like nicotine) could induce striatal dopamine release, SIB-1553A was significantly more effective than either SIB-1508Y or nicotine in evoking hippocampal acetylcholine release. Ultimately, SIB-1553A was earmarked primarily for the treatment of AD and SIB-1508Y was targeted to PD, the two syndromes manifesting respectively brain cholinergic and dopaminergic deficits. In early 1999, SIB-1508Y was evaluated in two phase II clinical trials, one as a combined regimen with other anti-PD agents and one as monotherapy for early stage PD. In mid-1999 a phase II trial of SIB-1553A was initiated in individuals with AD. The results of these studies have not yet been published.

Another example of targeting a synthetic nicotinic receptor agonist to a particular subtype of nicotinic receptor is represented by the compound GTS-21 [3-(2,4-dimethoxybenzylidene)anabaseine]. GTS-21 was patterned after the invertebrate toxin anabaseine, a nonselective nicotinic agonist. GTS-21 and its active metabolite 4-OH-GTS-21 have been characterized pharmacologically as partial agonists with preference (relative to nicotine) for the α_7 subtype of the nicotinic receptor [47]. Though the drug also interacts with equal or greater affinity to $\alpha_4\beta_2$ nicotinic receptors, it appears to display the functional characteristics of an α_7 nicotinic agonist. Despite its completely different subtype profile from the aforementioned nicotinic receptor agonists, GTS-21 also was shown to have cognitive enhancing actions with a low potential for side effects relative to nicotine. One clinical safety trial with GTS-21 in normal healthy males revealed that the compound was well tolerated and that the drug enhanced attention and working memory relative to placebo [48]. One issue that has yet to be resolved is which of the nicotinic receptor subtypes will prove most useful as a therapeutic treatment for disorders of cognition. For example, the compound TC-1734 [2*S*-4*E*-*N*-methyl-5-(5-isopropoxy-3-pyridinyl)-4-penten-2-amine], like GTS-21, is currently in phase II clinical trials for

cognitive enhancement in the elderly. The compound exhibited very good affinity for the $\alpha_4\beta_2$ subtype, but poor or no interaction with α_7 , ganglionic, and neuromuscular nicotinic receptor subtypes [49]. This laboratory's experience with many proprietary nicotinic receptor agonists as examined in monkeys performing the DMTS task would suggest that drugs more selective for the $\alpha_4\beta_2$ subtype produce a more reliable and predictable improvement in task accuracy relative to α_7 nicotinic agonists. However, drugs with α_7 selectivity appear to produce a more reliable and predictable neuroprotective action than compounds with $\alpha_4\beta_2$ selectivity. This impression requires confirmation and the study of subtype-selective drugs with enhanced specificity.

16.5 NEUROPROTECTIVE ASPECTS OF THERAPY WITH NICOTINIC RECEPTOR AGONISTS

Modern therapeutic approaches to the treatment of disorders of cognition like AD should include means to slow or halt the progress of the disease in addition to cognition enhancement. These two objectives could be addressed by a single molecular entity. Nicotine certainly possesses this dual potential. In fact, nicotine can increase the levels of neurotrophic factors and it increases the expression of nerve growth factor receptors in the brain [50]. These represent important properties of nicotine because basal forebrain cholinergic neurons are dependent upon a constant supply of neurotrophic substances for their maintenance and survival [51]. Nicotinic drugs also can protect neural cells against many types of cytotoxic insults, including that produced by amyloid proteins [52, 53]. The nicotinic α_7 receptor subtype appears to play an important role in nicotine's neuroprotective actions [52, 54], particularly in regions of the brain where the subtype is relatively highly expressed, for example, the temporal cortex and hippocampus (sites of early damage in AD). Support for the potential of synthetic nicotinic receptor agonists as disease-modifying agents in AD is derived from some clinical experience with the acetylcholinesterase inhibitor galantamine. Galantamine also can act as a noncompetitive agonist at nicotinic receptors, an action that has been suggested to provide the basis for the claim that the drug is able to sustain therapeutic benefit longer than the acetylcholinesterase inhibitors without this property [55]. Optimism regarding the therapeutic potential of α_7 receptor subtype agonists has been dampened by the linkage of α_7 receptor stimulation to an increase in the aggregation and phosphorylation state of tau. Tau in its hyperphosphorylated state is found in neurofibrillary tangles, one of the hallmark pathological entities associated with AD. However, the relevance of chronic doses of nicotine that have been used to induce excessive tau phosphorylation in mice [56], which are more than two orders of magnitude greater than that consumed by habitual cigarette smokers on a daily basis, to the clinical situation has yet to be established.

16.6 GLUTAMATE RECEPTOR LIGANDS

Like nicotinic acetylcholine receptors, ionotropic glutamate receptors are ligand-gated ion channels composed of various subunit combinations forming three major

classes: *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate receptors. There also is a separate major class of glutamate receptors that, like muscarinic receptors, are G-protein-linked metabotropic receptors [57]. Glutamate is the predominant excitatory neurotransmitter in the CNS, and the family of glutamate receptors plays a cooperative role in synaptic long-term potentiation (LTP). LTP is thought to represent a basic component of memory formation [58]. Therefore, glutamate neuronal systems have been suggested as targets for the development of new therapeutic approaches for the treatment of memory disorders, including AD. The NMDA receptor complex consists of a glutamate binding site, a strychnine-insensitive glycine coagonist site, a polyamine allosteric site, and two channel sites. In certain traumatic situations (e.g., reduced cerebral blood flow) glutamate accumulates at NMDA receptors, causing prolonged depolarization and eventually cell death. Kainic acid and other glutamate analogs (often termed excitotoxins) underscore the potential for toxicity associated with glutamate receptor stimulation [59]. However, compounds that act at the glycine site on the NMDA receptor complex can serve as procognitive agents based on their ability to enhance LTP without producing neurotoxicity. The glycine prodrug milacemide and the partial agonist/antagonist D-cycloserine enhance the performance of memory-related tasks in animal models [60] as well as in individuals with AD [61]. Another approach to limit the side-effect and toxicity profile associated with NMDA receptor agonists is exemplified with the development of drugs like IDRA 21 (7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine S,S-dioxide) that are allosteric modulators of the AMPA receptor. Unlike classical NMDA receptor agonists, IDRA 21 has been reported to produce neurotoxicity at concentrations far above its effective dose range as a cognitive enhancer [62]. Evidence garnered in favor of the mnemonic potential of IDRA 21 has largely focused on the ability of the drug to reverse pharmacologically induced amnesia or learning defects in normal animals. This laboratory had the opportunity to determine whether IDRA 21 was an effective cognitive-enhancing agent in normal young and aged rhesus monkeys [63]. IDRA 21 effectively increased task accuracies in monkeys with a sustained level of improvement that was apparent for at least 48 h after drug administration. Though we have noted pharmacokinetic–pharmacodynamic mismatches for a variety of cognition-enhancing drugs, protracted durations of action are not necessarily a general property of this class of agents [64]. However, in rats chronic administration of the AMPA modulator CX516 (Ampalex) improved memory-related task efficiency that persisted for an additional seven days after drug discontinuation [65]. The overall degree of effectiveness of IDRA 21 in the aged monkeys was not quite as robust as it was in young animals, and aged subjects also appeared to be more individually sensitive to drug dose. The ramifications of this finding for the potential treatment of individuals with AD or with respect to mnemonic processes in general are unclear.

Surprisingly it was an NMDA receptor antagonist that was the first of the glutamate drugs to show potential in clinical situations. Memantine is currently approved in the United States for the treatment of moderate to severe AD, and the drug is being prescribed in conjunction with acetylcholinesterase inhibitor therapy [66]. This compound is considered to be a disease-modifying agent. The theory is that the drug prevents the excitatory amino acid neurotoxicity suggested to accompany AD without interfering with the actions of glutamate required for learning and memory [67]. In fact, memantine has been reported to improve cognition and to

result in the early improvement in behavior in individuals with AD [68]. It is not clear whether these bonus therapeutic actions are the result of the drug's potential for slowing the disease process or whether there is a direct positive action on cognitive processes. Thus, whereas glutamate receptor agonists have the potential to improve cognitive abilities and memory, glutamate receptor antagonists have the potential to act as disease modifiers. Whether such compounds could act together in a treatment regimen for AD is unknown. Clearly receptor selectivity will be crucial for success in this situation.

16.7 OTHER POTENTIAL THERAPEUTIC TARGETS

Table 16.1 provides a partial listing of a variety of potential therapeutic targets that are presently under investigation for the treatment of disorders of cognitive function, including AD. At the start of this chapter the diversity of neural systems involved in cognition and memory was discussed. This anatomical and neurochemical diversity partly underlies the diversity of potential therapeutic targets mentioned above and listed in Table 16.1. It also is possible that several neural systems do not directly participate in mnemonic processes, but they impact the underlying mechanisms by participating in nonmnemonic pathways that mediate the influence of other physiological and behavioral states on memory. Irrespective of the mechanism of action, this diversity of potential therapeutic targets provides fertile ground for new drug development. This laboratory has had the opportunity to study the potential for many of these compounds in macaques in their performance of versions of the DMTS task. Figure 16.2 compares the effect of optimal doses of eight different therapeutic targets on task accuracies. Nicotinic receptor agonists represent the most extensive drug class because this target represents a long-standing interest of our laboratory. Within the nicotinic class there exists a wide degree of effectiveness. This also holds true for the three acetylcholinesterase inhibitors. Whereas a significant contribution to this intraclass variability is related to differences in nicotinic receptor subtype selectivity by the individual compounds, the differences noted for the acetylcholinesterase inhibitors are less obvious. However, acetylcholinesterase should not be considered a single drug target. Ample evidence exists to indicate that drugs that have selectivity for different major types of acetylcholinesterase—for example acetyl- vs. butyryl- (pseudo-) acetylcholinesterase—or which have selectivity for the different alleles of acetylcholinesterase have different pharmacological properties when it pertains to mnemonic efficacy and cholinergic side effects [69].

The data presented in Figure 16.2 illustrate a stepwise progression of drug efficacies in a single nonhuman primate model specifically designed to provide results that are predictive for human drug responsiveness. One important question is whether some ceiling effect has been reached for cognition-enhancing drugs in general or whether there is still room for improvement. Almost every major drug target is represented by at least one compound in the top third of the group. Within this group there is very little difference in positive mnemonic potential. The further improvement of drug efficacy within a class is a viable option; however, one possibility that deserves further study is that drugs that have multiple therapeutic targets will prove more effective than those with a single mechanism of action. Evidence for this possibility has recently been reviewed [70]. Two compounds designed to interact with more than one drug target

TABLE 16.1 Potential Cognition-Enhancing Drugs and Their Molecular Targets

Compound ^a	Target	Source	Pharmacological Action
PDE4 inhibitor	Phosphodiesterase-4/CREB	Helicon Therapeutics	Memory enhancement in young animals
SGS742	GABA _B (inhibitor)	SAEGIS Pharmaceuticals	Memory Enhancement consolidation in animals
CX516	Glutamate AMPA-R receptor	Cortex Pharmaceuticals	Improved cognition in clinical studies; MCI
ABT-239	Histamine H ₃ (antagonist)	Abbott Laboratories	Improved cognition in animal models
TC-1734	Central nicotinic cholinergic receptors	Targacept	Improved cognition in animal models; some clinical trials
AF102B	Muscarinic M ₁ cholinergic receptors	Israel Institute for Biological Research	Improved cognition in animal models; disease modification potential
NAP	Activity-dependent neuroprotective protein mimetic	Tel Aviv University	Improved spatial memory by rats
Ro 04-6790	5-HT ₆ receptor (antagonist)	Roche	Improved spatial memory by rats
Naloxone	Opiate receptors	Commercially available	Improved spatial memory by rats
AIT-082	Adrenal hormone receptors	NeoTherapeutics	Reversed cycloheximide-induced amnesia
Piribedil	D ₂ /D ₃ dopaminergic agonist	Commercially available	Enhanced cognitive skill learning in healthy older adults
JWS-USC-751X	Muscarinic M ₂ (antagonist)/acetylcholinesterase (inhibitor)	University of South Carolina	Improved cognition in animal models
TV3326	Monoamine oxidase/acetylcholinesterase (inhibitor)	TEVA Pharmaceutical Industries	Neuroprotectant; memory enhancement

^a *Abbreviations:* ABT-239 ([4-(2-{2-[(2R)-2-Methylpyrrolidinyl]ethyl}-benzofuran-5-yl)benzonitrile]); AF102B (Cevimeline); AIT-082 (Neotrofin); JWS-USC-751X (3-[[[2-[(5-dimethylaminomethyl)-2-furanyl]methyl]thio]ethyl]amino]-4-nitropyridazine); NAP (peptide NAPVSIPQ); PDE4 (phosphodiesterase-4); Ro 04-6790 (4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide); SGS742 (3-aminopropyl-n-butyl phosphinic acid); TC-1734 (Ispronicline); TV3326 (Ladostigil).

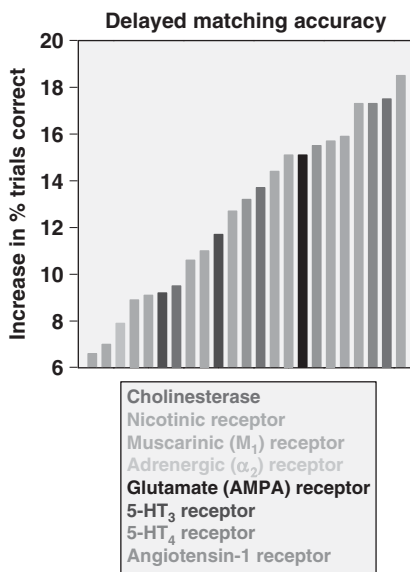


Figure 16.2 Increase in performance accuracies by macaques well trained in performance of computer-assisted DMTS task after administration of potential memory-enhancing agents. Drug-induced increases from baseline (vehicle) accuracy were obtained from data sets that included only the optimal dose in a series individualized for each subject (best dose) and that associated with most improved delay interval. All studies were performed on subjects maintained at the Medical College of Georgia Animal Behavior Center over the past 10 years. The drugs are coded to indicate the molecular target of the drug. (See color insert.)

appear in the last two rows of Table 16.1. One of these, JWS-USC-75IX, exhibits high potency as an inhibitor of both acetylcholinesterase and M₂ muscarinic acetylcholine receptors. A structural moiety for M₂ antagonism was added to the molecule to limit the presynaptic downregulation (reduced transmitter release) normally induced by acetylcholinesterase inhibitors. The drug produced a good profile of activity in memory-related tasks in rats [36]. Additional studies in monkeys will be required to determine to which segment of Figure 16.2 the drug will be allocated.

The compounds currently approved for the treatment of AD have already made an enormous impact on the lives of patients afflicted with this disease. They are far from optimal, and drug development in this area continues to be robust. Even if a means is developed to slow or even halt the progression of AD, it is likely that drugs designed to improve cognition and memory will continue to play an important role in medicine. The value of these drugs to the treatment of nondegenerative disorders of cognition or to improving memory in old age is soon to be realized. As mentioned above, it has been our experience that for the drugs that we have examined in young and aged monkeys there is little difference in drug efficacy between the two cohorts. Even now cognition-enhancing drugs are being consumed by young individuals looking for an edge during scholastic examinations or during job placement testing. Neither the risk/benefits under these circumstances nor the ethical ramifications of this behavior have been well studied.

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17

PHARMACOTHERAPY AND TREATMENT OF PARKINSON'S DISEASE

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17.1	Introduction	480
17.2	Pharmacotherapy of Parkinson's Disease: The Beginning	480
17.3	Neurochemistry of Parkinson's Disease	481
17.4	Treatment of Parkinson's Disease	483
17.5	Treatment of Motor Symptoms	484
17.5.1	Dopaminomimetic Agents	484
17.5.1.1	Levodopa Plus Peripheral Decarboxylase Inhibitor	484
17.5.1.2	Other Formulations of Levodopa	487
17.5.1.3	Dopamine Agonists	488
17.5.1.4	Monoamine Oxidase Type B (MAO _B) Inhibitors	494
17.5.1.5	COMT Inhibitors	495
17.5.2	Nondopaminomimetic Agents	496
17.5.2.1	Anticholinergic Drugs	496
17.5.2.2	Antiglutamate Agents	497
17.6	Treatment of Motor Complications	497
17.6.1	Wearing Off	498
17.6.2	Sudden "Off"	498
17.6.3	Delayed "On"	499
17.6.4	Dose Failure	499
17.6.5	Peak-Dose Dyskinesia	499
17.6.6	Diphasic Dyskinesia	499
17.6.7	Painful Dystonia	499
17.6.8	Yo-yo-ing	500
17.7	Treatment of Nonmotor Symptoms	500
17.7.1	Nausea and Vomiting	500
17.7.2	Orthostatic Hypotension	500
17.7.3	Depression and Anxiety	501
17.7.4	Dementia	502

17.7.5	Psychosis and Hallucinations	503
17.7.6	Other Nonmotor Symptoms	503
17.8	Neuroprotective Therapy	504
17.9	Treatment in the Near Future	505
17.10	Conclusion	506
	Acknowledgment	506
	References	506

17.1 INTRODUCTION

Parkinson's disease is one of the most common neurodegenerative disorders. Approximately one in a hundred persons of age 65 years and above are affected by the condition [1]. It is a movement disorder with cardinal features of tremor, rigidity, bradykinesia, and postural instability [2]. Secondary features include micrographia, hypophonia, hypomimia, gait disturbances, and freezing. Nonmotor features may be present, such as excessive drooling of saliva, constipation, and sleep disturbances.

While the exact etiology of Parkinson's disease is not known, the underlying pathology is related to the degeneration of dopaminergic neurons in the substantia nigra [3, 4]. The deficiency of striatal dopamine correlates well with the severity of bradykinesia but not with tremor [5]. Pathological changes of Parkinson's disease may be seen outside the nigrostriatal system, such as in the locus ceruleus, raphe nucleus, nucleus basalis of Meynert, limbic structures, and neocortex [6]. The involvement of these structures may account for some of the secondary and nonmotor features of Parkinson's disease.

The ultimate goal (challenge) in the treatment of Parkinson's disease is to identify agents that can stop or slow down the process of cell death. Unfortunately, none of the current available medications for Parkinson's disease are convincingly neuroprotective. Several factors are implicated in the process of cell death, and efforts in searching for neuroprotective agents are ongoing. There were also attempts to restore dopaminergic neurons through transplantation [7, 8] and the infusion of neurotrophic factors [9]. To date, the established treatment of Parkinson's disease is still symptom control and the management of the complications of treatment. These underlie the principles of pharmacotherapy in Parkinson's disease. The role of surgery in Parkinson's disease is beyond the scope of this chapter.

17.2 PHARMACOTHERAPY OF PARKINSON'S DISEASE: THE BEGINNING

James Parkinson first described Parkinson's disease in 1817 [10], but effective treatment was not available until five decades later. Ordenstein [11] and Charcot [12] in Paris were the first to use plant extracts that contain anticholinergic compounds such as hyoscyne and scopolamine to treat Parkinson's disease. By the 1940s, numerous synthetic anticholinergic compounds were available. These relieved the symptoms of tremor and rigidity, but the effects were at most modest. The

breakthrough came in 1959 when Carlsson [13] argued that dopamine is an important neurotransmitter in the basal ganglia. A year later, Ehringer and Hornykiewicz [3] showed that dopamine levels are reduced in the brains of patients with Parkinson's disease. This was followed later by reports on improvement of rigidity and bradykinesia following an intravenous or oral dose of levodopa, a dopamine precursor [14–16]. Levodopa has to be given instead of dopamine because the latter does not cross the blood–brain barrier. Unfortunately, peripheral conversion of levodopa to dopamine occurs, and this contributes to side effects and reduces the amount of levodopa delivered to the brain. In 1967, Cotzias and coinvestigators [17] demonstrated the efficacy of high-dose oral levodopa and the improvement of side effects following coadministration of a peripheral decarboxylase inhibitor, which reduces the peripheral conversion of levodopa to dopamine. Since then, the combination of levodopa and peripheral decarboxylase inhibitor has proven to be an effective treatment of Parkinson's disease. Newer drugs were developed over the past three and a half decades, but none is superior to levodopa in terms of antiparkinsonian effects. Levodopa is still the “gold standard” treatment of Parkinson's disease to date.

17.3 NEUROCHEMISTRY OF PARKINSON'S DISEASE

The underlying biochemical abnormality in Parkinson's disease is deficiency of dopamine [3]. The main dopamine-producing nuclei in the brain are the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). The SNc terminals project to the striatum to form the nigrostriatal pathway that is integral to the motor loop of the basal ganglia. The VTA terminals project to the limbic and cortical structures (mesocorticolimbic pathway), and these modulate behavioral and cognitive functions. In Parkinson's disease, the nigrostriatal pathway is affected. Parkinsonian features appear when 80% of the striatal dopamine or 50% of the nigral cells are lost [4, 18].

Endogenous dopamine is synthesized within nerve terminals of dopaminergic neurons (Fig. 17.1). Tyrosine is first converted to L-3,4-dihydroxyphenylalanine (levodopa) by the enzyme TH. Levodopa is then decarboxylated by AADC to dopamine. The synthesized dopamine is stored in presynaptic vesicles. Following depolarization of nerve terminals, the stored dopamine is released into the synaptic cleft and interacts with the pre- and postsynaptic dopamine receptors.

There are five types of dopamine receptors (D_1 to D_5), divided into two broad families: D_1 and D_2 families [19–21]. The D_1 family of receptors (D_1 and D_5) are positively linked to adenylate cyclase [19]. The D_2 family of receptors (D_2 , D_3 , and D_4) are negatively linked to adenylate cyclase [22]. The major dopamine receptors in the striatum are D_1 and D_2 receptors. The other dopamine receptors are more common over the limbic and cortical areas.

Enzymes such as MAO and COMT metabolize dopamine to DOPAC and HVA. The extracellular dopamine is also transported back into the presynaptic terminal by the dopamine transporters. Once inside, the dopamine is either recycled into the presynaptic vesicles or undergoes metabolism by intracellular MAO to DOPAC. The latter diffuses out of the cell and is further metabolized by COMT to HVA.

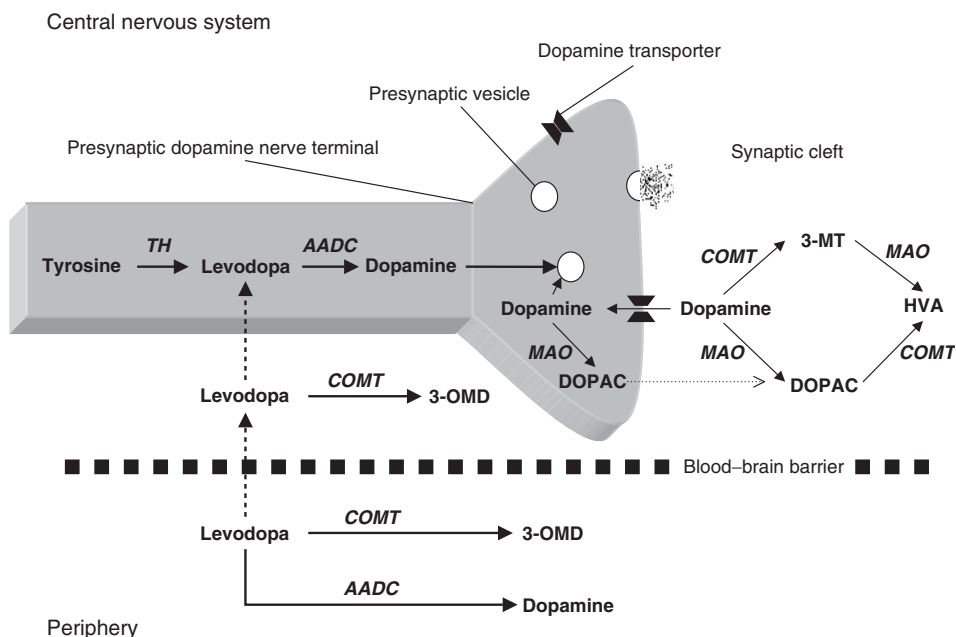


Figure 17.1 Dopamine synthesis and metabolism: AADC, aromatic amino acid decarboxylase; COMT, catechol-*O*-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MAO, monoamine oxidase; TH, tyrosine hydroxylase; 3-MT, 3-methoxytyramine; 3-OMD, 3-*O*-methyldopa.

In Parkinson's disease, there are reduced levels of TH, AADC, dopamine, and HVA [3, 23–25]. Besides dopamine, the other monoamines may also be affected. There are reports of reduced noradrenaline and serotonin levels in the forebrain regions of patients with Parkinson's disease [26, 27]. The locus ceruleus is the major source of noradrenaline in the brain [28], and the raphe nucleus is the principal source of serotonin [29]. Both structures are affected in Parkinson's disease [6].

The motor loop of the basal ganglia is rather complex [30]. The striatum receives afferent inputs from the cortex, thalamus, SNc, and raphe nucleus. The basal ganglia in turn send efferent projections to the thalamus and brain stem. Multiple interconnecting neurons are present within the basal ganglia. There are also projections from the raphe nucleus and locus ceruleus to the substantia nigra pars reticularis (SNr). There are two major pathways in the motor loop: direct and indirect pathways. In the direct pathway, the striatum projects directly to the globus pallidus interna and SNr. In the indirect pathway, the projections to these structures are indirectly via the globus pallidus externa and subthalamic nucleus. Different neurotransmitters are present at different levels, and these include dopamine, acetylcholine, γ -aminobutyric acid (GABA), glutamate, enkephalin, dynorphin, and substance P (Fig. 17.2). The glutamate pathways are excitatory, and the GABA pathways are inhibitory. The action of dopamine on D_1 receptors in the striatum (direct pathway) is excitatory, and the action on D_2 receptors (indirect pathway) is inhibitory. Under normal circumstances, stimulation of D_1 and D_2 receptors in the striatum reduces the GABA outflow of globus pallidus interna and

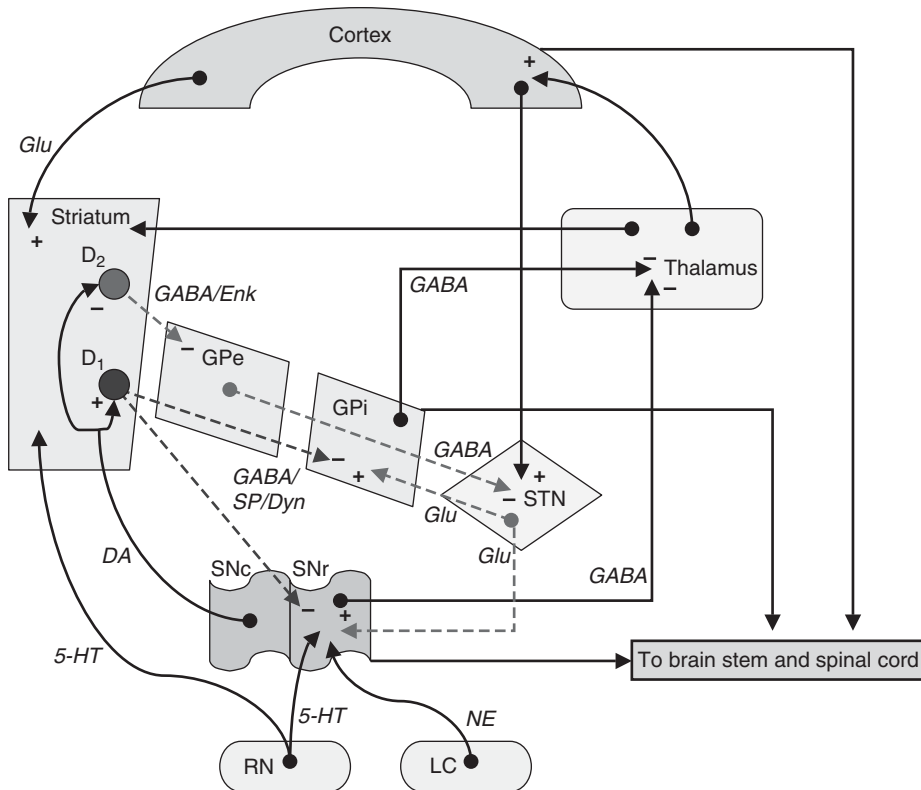


Figure 17.2 Schematic of basal ganglia motor loop; direct and indirect pathways: Dashed line, direct pathway; dotted line, indirect pathway; +, excitatory stimulation; −, inhibitory stimulation; D₁, D₁ receptors; D₂, D₂ receptors; GPe, globus pallidus externa; GPi, globus pallidus interna; STN, subthalamic nucleus; RN, raphe nucleus; LC, locus ceruleus; DA, dopamine; Glu, glutamate; 5-HT, serotonin; NE, noradrenaline; Enk, enkephalin; SP, substance P; Dyn, dynorphin. (See color insert.)

SNr. The inhibitory influence on the thalamus is reduced and there is an increase in thalamocortical activity. In Parkinson's disease, reduced dopamine activation of striatal D₁ and D₂ receptors will result in reduced thalamocortical activity and hence development of bradykinesia and other parkinsonian features.

The striatum contains both medium spiny neurons (GABAergic) and large aspiny neurons (cholinergic) [31]. Reduced dopaminergic activity results in dysregulation of GABA function and cholinergic overactivity. There are also adenosine A_{2A} receptors within the striatum. The A_{2A} receptors interact with the D₂ receptors at the signal transduction level [32].

17.4 TREATMENT OF PARKINSON'S DISEASE

The current medications used in treating Parkinson's disease provide only symptom control. They do not stop or prevent the underlying neurodegenerative process.

The medications are also associated with side effects that ultimately limit their use. Treatment should therefore be tailored according to the patient's symptoms, the degree of functional impairment, the expected therapeutic benefits, and the potential side effects of medications. We may delay pharmacotherapy in the early stages of the disease, when the symptoms are mild and do not interfere with functions. We normally treat the symptoms when the patient's functional level is impaired even though the symptoms are mild.

Table 17.1 lists the current medications available in treating Parkinson's disease. These may be divided into those that treat the motor symptoms of Parkinson's disease, and those that treat the nonmotor features. Knowledge on the pharmacology of these medications will help us to choose the appropriate agent(s) to provide the best symptom control with minimal side effects.

17.5 TREATMENT OF MOTOR SYMPTOMS

Most of the therapeutic agents that relieve the motor symptoms of Parkinson's disease act through the dopaminergic system. Examples are levodopa, dopamine agonists, COMT inhibitors, and MAO type B inhibitors. There are also drugs that act outside the dopaminergic system and these include the anticholinergic and antilutamate agents.

17.5.1 Dopaminomimetic Agents

17.5.1.1 Levodopa Plus Peripheral Decarboxylase Inhibitor. Levodopa was introduced in the late 1960s and is still the "gold standard" treatment. It relieves most motor features, in particular bradykinesia. The effects are so dramatic that the response to levodopa is used to differentiate Parkinson's disease from other akinetic-rigid conditions [33]. Its effects on tremor and postural instability are less remarkable.

Levodopa is a dopamine precursor that is absorbed in the small intestines through the neutral amino acid transport system [34]. Once absorbed, it is distributed widely into other tissues. It undergoes extensive first-pass metabolism, and less than 5% of the oral dose of levodopa reaches the brain [35]. The peripheral AADC and COMT convert levodopa to dopamine and 3-OMD, respectively (Fig. 17.1). Levodopa is given together with a peripheral decarboxylase inhibitor, such as carbidopa, to minimize nausea by decreasing circulating dopamine (see below). To block most of the peripheral decarboxylase activity, a daily dose of 75 mg or higher of carbidopa is required [36, 37]. Levodopa crosses the blood-brain barrier through the same transport system as in the small intestines. The 3-OMD may compete with levodopa at this level, further reducing the transport of levodopa to the brain [38, 39]. There is now a "3-in-1" tablet that contains levodopa, a peripheral decarboxylase inhibitor, and a COMT inhibitor to help overcome this problem [40]. Once inside the brain, the levodopa is converted to dopamine by the central AADC. The synthesized dopamine then exerts its effects and undergoes metabolism.

There are two types of peripheral decarboxylase inhibitors available: carbidopa and benserazide. In United States and Canada, the levodopa-carbidopa preparation (Sinemet) is commonly used. In some other countries, the levodopa-benserazide preparation (Madopar) is more often prescribed. Standard Sinemet is available in

TABLE 17.1 Medications Currently Available for Treating Parkinson's Disease

Drugs	Indications
<i>Dopaminomimetic Agents</i>	Motor symptoms. See text for details.
Levodopa/peripheral decarboxylase inhibitor	
Standard preparations	
Levodopa/carbidopa (Sinemet)	
Levodopa/benserazide (Madopar)	
Slow-release preparations	
Levodopa/carbidopa (Sinemet CR)	
Levodopa/benserazide (Madopar HBS)	
Dual-release preparation: levodopa/benserazide (Madopar DR)	
Dispersible preparation: levodopa/benserazide (Madopar Dispersible)	
Dopamine agonists	
Ergot derivatives	
Bromocriptine	
Pergolide	
Lisuride	
Cabergoline	
Dihydroergocryptine	
Nonergot derivatives	
Pramipexole	
Ropinirole	
Piribedil	
Apomorphine	
MAO _B inhibitors	
Selegiline	
Rasagiline	
COMT inhibitors	
Tolcapone	
Entacapone	
Levodopa/carbidopa/entacapone (Stalevo)	
<i>Nondopaminomimetic Agents</i>	Motor symptoms. See text for details.
Anticholinergic agents	
Trihexyphenidyl	
Bentropine	
Antiglutamate agent: amantadine	
<i>Agents That Relieve Nonmotor Symptoms</i>	
Domperidone, trimethobenzamide (Tigan)	Nausea and vomiting
Fludrocortisone, midodrine	Orthostatic hypotension
Laxatives	Constipation

TABLE 17.1 (*Continued*)

Drugs	Indications
Anticholinergic agents, e.g., oxybutynin	Detrusor hyperactivity
Tricyclic antidepressants, selective serotonin reuptake inhibitors, atypical antidepressants	Depression
Anxiolytics	Anxiety
Cholinesterase inhibitors	Dementia
Atypical neuroleptics	Hallucinations and psychosis
Clonazepam	Rapid-eye-movement (REM) sleep behavior disorder
Modafinil	Excessive daytime sleepiness

tablet strengths of 100/10, 100/25, and 250/25. The first number denotes the amount of levodopa in milligrams and the second number denotes the amount of carbidopa in milligrams. Madopar comes in a 4:1 ratio of levodopa to benserazide. Madopar 250 contains 200 mg of levodopa and 50 mg of benserazide.

The pharmacokinetics and pharmacodynamics of Sinemet and Madopar are similar [41]. The time to peak plasma concentration after a standard dose of levodopa–peripheral decarboxylase inhibitor is 30–60 min. The plasma elimination half-life is between 1 and 2 h. The bioavailability of levodopa may be affected by factors such as the speed of gastric emptying and the amount of protein in the diet [35]. Food and acidity in the stomach slow down gastric emptying and prolong the time taken to reach plasma concentration of levodopa. Protein in the diet interferes with absorption of levodopa by competing for the same transport system [42]. For maximal absorption of levodopa, patients can take the medication on an empty stomach either one-half hour before meals or 1–2 h after meals. While this is common practice there are those who advocate taking levodopa with a light meal or a snack to minimize side effects (see below), prolong duration of action, and promote compliance.

The most common side effect of levodopa is nausea [17, 43–46]. This is due to the action of peripheral dopamine on the chemoceptor trigger zone [47], which is located in the brain stem but outside the blood–brain barrier. The peripheral decarboxylase inhibitor in the standard dose of Sinemet and Madopar does not completely inhibit the peripheral conversion of levodopa to dopamine. To circumvent this problem, Sinemet or Madopar is initiated with a small starting dose and patients are advised to take the medication with a light meal or snack to delay gastric emptying. This reduces the speed of absorption of levodopa and hence reduces the intensity of the side effects. The dose is gradually increased, and after some time patients generally develop tolerance to the nausea. Most patients require 500–800 mg of levodopa daily. In some patients, a dose of 1500 mg or greater may be needed.

Other side effects include orthostatic hypotension [46, 48], arrhythmia [46, 49], hallucinations [44], and psychosis [50]. Some patients may develop a hypersexual response [45, 51, 52] and a compulsion for gambling [53]. Some of these side effects are related to dopamine actions on the peripheral and extrastriatal dopamine

receptors. Other less common side effects include an altered taste sensation, blurred vision, gastrointestinal ulcer [43], asthenia, and fatigue [44]. Abnormal laboratory results such as agranulocytosis, anemia [54], thrombocytopenia [55], elevated serum urea [56], and glucose levels [57] have been reported but are very uncommon. There were suggestions of increased risk of developing or reactivating melanoma with levodopa, since the synthesis of dopamine and melanin shares a common pathway [58–61]. Subsequent reports, however, suggested that the occurrence might be coincidental rather than causative [62–64]. In fact, levodopa may have an antitumor effect on melanoma [65]. The current consensus is that levodopa can still be given to patients with Parkinson's disease who have a history of melanoma. However, close dermatological observation is indicated.

Another side effect of chronic levodopa therapy is the development of dyskinesia and motor fluctuations. It is estimated that 20–50% of all patients treated with levodopa for three to five years develop motor fluctuations [44, 66–72]. The pathophysiology of this complication is not understood. It has been postulated that the dying dopamine neurons have reduced buffering capacity to handle the exogenous dopamine load [73]. Another suggestion is that the pulsatile stimulation of dopamine receptors leads to downstream receptor changes that ultimately cause motor fluctuations [74, 75]. Finally, “wearing-off” phenomenon may be related to increased dopamine turnover [76].

At one time levodopa was thought to be neurotoxic. In vitro studies showed that oxidized metabolites of levodopa (such as hydrogen peroxide and quinones) [77] are neurotoxic in high concentrations [78, 79]. Animal studies, however, did not reveal any detrimental effects of chronic levodopa therapy [80–82]. In the ELLDOPA study [83], in which the effects of levodopa on clinical progression of Parkinson's disease were studied, the levodopa treatment group had a slower rate of clinical progression compared to the placebo group, and the effects were sustained even after two weeks of washout period. The washout period of levodopa has been reported to be as long as six weeks [84].

17.5.1.2 Other Formulations of Levodopa. The plasma elimination half-life of the standard Sinemet or Madopar is approximately 1–2 h [41]. In an attempt to provide sustained clinical effects of levodopa with fewer fluctuations in plasma levels, slow-release formulations were introduced.

Sinemet CR (controlled release) comes in a matrix tablet that releases the active ingredients slowly through surface dissolution and erosion as it passes along the small intestine [85]. Madopar HBS (hydrodynamically balanced system) comes in a capsule that is transformed into a gelatinous diffusion body upon gastric contact [86]. The gelatinous material floats in the stomach for 3–6 h. Both Sinemet CR and Madopar HBS may extend the plasma elimination half-life of levodopa by up to 2 h compared to the standard formulations. Although some studies claimed that patients with Parkinson's disease may be maintained on a twice-daily regimes of Sinemet CR, in clinical practice benefit is not sustained for longer than 6 h, and a three- to four-times daily dosing is still required [87, 88]. The slow-release formulations provide a more stable plasma level of levodopa with frequent dosing, but these do not prevent the development of motor fluctuations [44, 89]. Crushing will destroy the slow-release properties of Sinemet CR; however, tablets can be cut in half on a

physician's order. These drugs should be taken with food, as this will increase the bioavailability by 50% and the peak plasma concentration by 25% [85]. The time to peak plasma concentration of the slow-release formulation is 2 h. The bioavailability is 25–30% lower than the standard preparations. When converting standard Sinemet to Sinemet CR, higher dosing is required. A single dose of Sinemet CR 200/50 is equivalent to 140–150 mg of standard Sinemet.

In Switzerland, there is a dual-release formulation of levodopa (Madopar DR) that consists of a three-layer tablet with both immediate- and slow-release properties [90]. It provides a more rapid onset of action compared to the slow-release formulations but at the same time provides longer clinical effects compared to standard levodopa. Other formulations of levodopa include dispersible preparations [91] and water-soluble ethyl ester preparations [92]. The dispersible preparations form a suspension with water that allows rapid absorption of levodopa. The ethyl ester tablets are highly water soluble and rapidly hydrolyzed in the stomach to release levodopa.

17.5.1.3 Dopamine Agonists. Dopamine agonists are a group of drugs that act directly on the dopamine receptors without the need for *in vivo* conversion to dopamine. They do not compete with amino acids for absorption in the gastrointestinal tract and crossing the blood–brain barrier. Bromocriptine was the first dopamine agonist introduced for treatment of Parkinson's disease [93]. Since then, a number of other dopamine agonists have been developed. They have diverse pharmacokinetic and pharmacodynamic profiles, but all of them act on the D₂ family of receptors. As a group, dopamine agonists are inferior to levodopa in terms of their level of efficacy [67, 69, 94–97]. However, they cause less dyskinesia and have a more prolonged action. They also lower the dose requirement of levodopa. Dopamine agonists have been used as adjunctive therapy to levodopa in advanced Parkinson's disease. In recent years, there has been a trend to use them as monotherapy in the early stages of the disease, especially in younger patients [98]. The rationale is to reduce the levodopa requirement, thus possibly decreasing the risk of developing levodopa-induced motor complications.

Table 17.2 lists the dopamine agonists currently available. Most of the newer agonists were compared to bromocriptine. Their efficacy and side-effect profiles were similar, although subtle differences may favor one agonist over another. A switch from one agonist to another can be made by substituting a bioequivalent dose [101, 103, 107]. The conversion ratios of the various agonists are given in Table 17.2.

17.5.1.3.1 Bromocriptine. Bromocriptine is an ergot derivative with potent D₂ agonistic effect and mild D₁ antagonistic effect. It was first introduced as a treatment of Parkinson's disease in 1974 [93]. It is effective as an adjunctive therapy to levodopa in patients with advanced Parkinson's disease and motor fluctuations [108–111]. It may also be used as a monotherapy [112–114]. Patients treated with bromocriptine have a lower incidence of dyskinesia [68, 69, 115]. However, it is inferior to levodopa in terms of antiparkinsonian effects [68, 69]. Most dopamine agonists have comparable efficacy to bromocriptine [116–119].

Bromocriptine comes in two preparations: a 2.5-mg tablet and a 5-mg capsule. It is absorbed from the gastrointestinal tract and undergoes first-pass metabolism in the

TABLE 17.2 Pharmacology of Dopamine Agonists

Medications	Route	$t_{1/2}$ ^a (h)	Daily dose (mg/day)	Conversion Ratio ^b	Receptor Affinity ^c							
					D ₁	D ₂	D ₃	D ₄	D ₅	5-HT	α_1	α_2
Ergot derivatives												
Bromocriptine	Oral	3–8	20–40	10	±	++	++	+	+	++	+++	++
Pergolide	Oral	7–27	1.5–3	1	+	++	+++	++	++	+++	+	++
Lisuride	Oral	2–3	1–5	1	+ / 0	++++	++++	+++	+++	+++	+++	++++
Cabergoline	Oral	65–110	2–6	1.3	+	++++	++++	++	++	+++	+	++
Dihydroergocryptine	Oral	15	30–120	40	±	+++	?	?	?	?	?	?
Nonergot derivatives												
Pramipexole	Oral	8–12	2–4.5	1	0	+	+++	++	0	0 / + (5-HT ₁)	0	0 / +
Ropinirole	Oral	3–6	3–24	4	0	+	++	+	0	0 / + (5-HT _{1A} , 5-HT _{1D} , and 5-HT _{2B})	0	0 / +
Piribedil	Oral	21	150–250	50–100	0	+	+	+	0	0 / + (5-HT _{1A} and 5-HT _{2B})	+	++
Apomorphine	Subcutaneous	0.5	3–30	—	+	++	++	+++	++	+	0 / +	++

^aHalf-life

^bConversion ratio, with pergolide as unity.

^cD₁ to D₅, dopamine receptors; 5-HT, serotonin receptor; α_1 , α_1 -adrenergic receptor; α_2 , α_2 -adrenergic receptor; 0, no affinity; +, low affinity; ++, moderate affinity; +++, high affinity; +++++, very high affinity; ?, not known; \pm , partial agonist or mild antagonist. Data based on [41, 99–107].

liver [41]. The bioavailability is less than 10% of the oral dose. The time to peak plasma concentration is between 1 and 3 h, and the plasma elimination half-life ranges from 3 to 8 h [102]. Bromocriptine is subject to drug interactions. Hepatic enzyme inhibitors such as macrolides may increase plasma bromocriptine concentration [120].

Bromocriptine is associated with side effects similar to those of levodopa [68, 69, 108, 115]. Its action on dopamine receptors may cause nausea, postural dizziness, hallucinations, and psychosis. Other neuropsychiatric side effects include agitation, depression, confusion, nightmares, and insomnia. Some of these effects may be due to its action on serotonergic and adrenergic receptors. It may rarely cause erythromelalgia—bilateral, red, hot, and painful feet [108, 121], pleural fibrosis [122], and retroperitoneal fibrosis. The reactions are dose dependent and reversible upon termination of drug therapy.

The side effects are better tolerated if the starting dose of bromocriptine is small and gradually increased [123]. The average dose requirement is between 20 and 40 mg daily. In general, the side effects are less frequent at lower doses of bromocriptine (30 mg or less daily) and occur more frequently with higher doses (50 mg and above daily).

17.5.1.3.2 Pergolide. Pergolide is a synthetic semi ergot compound that was introduced in 1988. It is a potent D₂ and D₃ agonist with mild D₁ agonistic effects [41]. It also has actions on adrenergic and serotonergic receptors [124, 125]. Pergolide is effective either as a monotherapy [126] or as an adjunctive therapy to levodopa [127]. There are theoretical advantages of D₁ actions on cognition [128], bladder function [129], and fatigue [130]. There is no convincing evidence, however, that D₁ stimulation is achieved, in clinical practice, by the tissue concentration of pergolide.

The time to peak plasma concentration is between 1 and 3 h [41]. It undergoes extensive first-pass metabolism and is completely eliminated within five days. Pergolide is available in 0.05-, 0.25-, and 1 mg-tablets. The average maintenance dose of pergolide is 3 mg daily, although Asians tend to tolerate a lower dose range (0.75–1.25 mg daily in Japan) [131]. There have been reports that a higher dose range of 5 mg and above daily may be needed to observe the optimal benefits from pergolide [132, 133]. The main concern is the risk of side effects.

The common side effects of pergolide are the same as with most dopamine agonists. Nausea, postural dizziness, leg edema, cardiac arrhythmia, and somnolence have all been reported [126, 127, 134, 135]. Due to its ergot properties, it also leads to dose-dependent pleural and retroperitoneal fibrosis [136]. One of the more significant side effects that has come to light recently is the problem of restrictive valvular heart disease [137, 138]. During the early years when pergolide was introduced, the incidence of cardiac valvulopathy was reported to affect 1 in 20,000 patients treated. However, using echocardiography, it was shown that up to one-third of patients treated with pergolide (26 of 78 patients treated) had restrictive valvular heart disease [138]. None of the 18 patients in the placebo arm, who had never been treated with an ergot-derived dopamine agonist, was affected. The incidence was higher in those treated with 5 mg or more of pergolide (42% of 26 patients) compared to those with a lower dose (29% of 26 patients). In 6 patients where pergolide was stopped, there was regression of the valve disease, suggesting that this side effect may potentially be reversible. In a separate study by Baseman et al. [137], 89% of 41 patients treated with pergolide had some degree of valvular insufficiency. When compared to an age-

matched control group derived from the Framingham Study, the risk of having abnormal valves in pergolide-treated patients was increased by two- to three-fold. Tricuspid regurgitation was the more common pathology. There are still unresolved issues whether this side effect is peculiar to pergolide, a class effect with ergot-derived dopamine agonists, or an effect through the serotonin 5-HT_{2B} receptors. Literature on appetite-suppressant drugs suggests drug-induced fibrotic valvular heart disease may be linked to the serotonin receptors [139–141]. Pergolide interacts with serotonin receptors [141], as do some other dopamine agonists [125].

17.5.1.3.3 Lisuride. Lisuride is an ergot-based dopamine agonist that was introduced in 1990. It is not available in the United States or Canada but is marketed as both monotherapy and adjunctive therapy to levodopa in several European countries [96, 142]. Its main actions are on the D₂ family of receptors, with minimal or no effect on D₁ receptors. Its efficacy is comparable to bromocriptine [118]. As with most other dopamine agonists, it has interactions with serotonergic and adrenergic receptors [124, 125]. The oral bioavailability of lisuride is low due to its extensive first-pass metabolism [41]. Its plasma elimination half-life is around 2 h, and the time to peak plasma concentration is between 60 and 80 min. Lisuride comes in a 200 µg tablet and the dose is increased gradually by 200 µg every week to a maximum of 5 mg daily in three divided doses. The side effects are comparable to those of bromocriptine [118]. However, the psychiatric complications are more frequent in patients treated with lisuride than in patients treated with bromocriptine [118] or dihydroergocryptine [143]. Lisuride is water soluble and may be given intravenously or subcutaneously. Continuous lisuride infusion can be beneficial in patients with advanced Parkinson's disease and motor fluctuations, but this route is limited by the high incidence of psychiatric side effects and the technical inconvenience of using an infusion pump [144–146]. Transdermal delivery of lisuride appears promising [147].

17.5.1.3.4 Cabergoline. Cabergoline is the only dopamine agonist that has a really long plasma elimination half-life (65–110 h), allowing it to be taken only once daily [102]. Like pergolide, it is an ergot-derived compound with strong D₂ actions and weak D₁ effects. It was introduced in 1997, available mainly in Europe and Japan. It is not available in the United States or Canada. Its role as a monotherapy remains unclear. After three to five years of treatment, most patients need levodopa therapy [148]. However, it is clinically useful as an adjunctive therapy to levodopa in advanced Parkinson's disease with motor fluctuations [149]. Its long plasma elimination half-life is a double-edged sword. While it allows once-daily dosing, it takes longer to washout side effects. The side effects of cabergoline are the same as any other ergot-derived dopamine agonist [116]. Cabergoline is available in 1-, 2-, and 4-mg tablets. The starting dose is 0.5–1 mg daily and may be increased by 0.5–1 mg every one to two weeks. The average dose requirement is 2–6 mg daily.

17.5.1.3.5 Dihydroergocryptine. Dihydroergocryptine has D₂ agonistic and partial D₁ agonistic effects. It is available in a few countries in Europe. Its oral bioavailability is below 5%, and its pharmacodynamic profile is comparable to that of bromocriptine. Clinical studies on dihydroergocryptine are limited. So far, it has been reported to have comparable efficacy to bromocriptine and lisuride [99].

17.5.1.3.6 Pramipexole. Pramipexole is a nonergot dopamine agonist that has good affinity for the D₂ family of receptors with negligible D₁ and serotonergic effects [41]. It was introduced in 1997 as both effective monotherapy [95, 150, 151] and adjunctive therapy to levodopa [152–155]. There have been reports of pramipexole having superior antitremor properties compared to placebo [156, 157]. When compared to pergolide, the antitremor properties were similar, although patients were more likely to discontinue pergolide than pramipexole because of side effects [158]. Compared to levodopa, the improvement in total and motor UPDRS (Unified Parkinson's Disease Rating Scale) scores from baseline to 48 months was greater in the levodopa treatment group than in the pramipexole group [150]. There were also higher frequencies of somnolence and leg edema with pramipexole compared to levodopa [150]. There have been reports of pramipexole having antidepressant properties [159, 160], and this may be achieved through the mesolimbic dopaminergic pathway [161]. The evidence for a neuroprotective effect is unconvincing [162, 163].

The side effects of pramipexole have been comparable to bromocriptine in terms of the gastrointestinal, cardiovascular, and neuropsychiatric complications [152]. The occurrence of fibrosis has not yet been reported. Hypersomnolence and “sleep attacks” have been reported and have led to some concerns regarding safety when driving [164, 165]. Somnolence is common in patients taking greater than 1.5 mg of pramipexole per day. Before initiating treatment with pramipexole, patients should be warned of this side effect. In some countries, patients are advised not to drive while taking pramipexole. The issue of somnolence, however, is not specific to nonergot compounds and may be seen in other dopamine agonists and levodopa monotherapy [166–170]. Parkinson's disease itself is also associated with sleep disturbances [171, 172].

Pramipexole comes in 0.125-, 0.25-, 0.5-, 1-, and 1.5-mg tablets. It has excellent bioavailability of 90%, with a plasma elimination half-life of 8–12 h and time to peak plasma concentration of 1–3 h [41]. It is eliminated largely unchanged in the urine. As such, it should be given with caution in patients with renal failure. The average dose is 2–4.5 mg daily in three or four divided doses.

17.5.1.3.7 Ropinirole. Ropinirole is another nonergot compound that has been available since 1997. It is specific for the D₂ family of receptors and especially the D₃ receptors [104]. It has little or no effects on the other receptors, but it may still interact with 5-HT_{2B}, 5-HT_{1A}, and 5-HT_{1D} receptors [105]. Although ropinirole is a nonergot compound, there was a recent report of fibrotic reactions [173]. Ropinirole otherwise has a similar side-effect profile to other dopamine agonists, such as nausea, postural dizziness, leg edema, and hallucinations [71, 117]. In fact, neuropsychiatric complications were of similar frequencies to bromocriptine [117], and hallucinations were more frequent with ropinirole than levodopa [71]. There was also a higher incidence of somnolence with ropinirole, as with pramipexole [164, 174].

As with most dopamine agonists, ropinirole is used as both monotherapy and adjunctive therapy to levodopa [71, 117, 175, 176]. Its antiparkinsonian effects are inferior to those of levodopa. At the end of three to five years, less than 20% of the patients were maintained on ropinirole monotherapy. It has been claimed that ropinirole might slow down the progression of Parkinson's disease [97], but the evidence is unconvincing [162, 163].

Ropinirole is available in 0.25-, 0.5-, 1-, 2-, 3-, 4-, and 5-mg tablets. Its bioavailability is between 46 and 55% with plasma elimination half-life of 3–6 h

[102]. It is usually prescribed in three to four divided daily doses. The time to peak plasma concentration is around 90 min. It undergoes metabolism in the P450 cytochrome system and may be subject to drug interactions. The therapeutic dose range is 3–24 mg daily.

17.5.1.3.8 Piribedil. Piribedil is used mainly in Europe and Asia. It is a nonergot compound with D₂ and D₃ agonistic actions and α_2 antagonistic effects [177]. Piribedil is supposedly effective in controlling tremor [178, 179] postural instability, and gait disturbances [180], possibly through its α_2 antagonistic effects. Repeated administration of piribedil induced less dyskinesia than levodopa in MPTP-treated monkeys [181].

Piribedil comes in a 50-mg sustained-release tablet with a long plasma elimination half-life of 21 h [100]. The time to peak plasma concentration is 1 h [102]. It is titrated to one tablet three times daily when used as an adjunctive therapy to levodopa. The dose may be increased to one tablet five times daily when used as a monotherapy. The side effects are similar to other dopamine agonists, and sleep attacks have also been reported [166].

17.5.1.3.9 Apomorphine. Apomorphine is a dihydroaporphine, derived by reducing morphine with hydrochloric acid and then oxidizing it by contact with air. It is a potent dopamine agonist, with high affinities for both D₁ and D₂ families of receptors [41]. It also has moderate affinities for serotonergic and adrenergic receptors.

Apomorphine was first observed to have antiparkinsonian effects by Schwab et al. in 1951 [182]. It was not widely used then because of its side effects. In 1979, Corsini et al. [183] showed that domperidone, a peripheral dopamine antagonist, could reduce the side effects of apomorphine. Since then, apomorphine has been used in Europe, mainly as a rescue agent for intractable motor fluctuations. It has only recently been approved for use in the United States to treat sudden, unexpected, and resistant “off” phenomena that do not respond well to current antiparkinson medications.

Apomorphine has a very high first-pass effect and cannot be given orally. It is administered via the subcutaneous route. It comes in ampoules or prefilled pen injectors with solution strength of 10 mg/mL. The time to peak plasma concentration is around 10 min, and its plasma elimination half-life is approximately 30 min [106]. The clinical response is seen within 5–15 min following subcutaneous injection, and the effect may last up to 90 min, with shorter duration of response in severe cases. Apomorphine reduces “off” time by more than 50%. It has clinical efficacy comparable to that of levodopa [184].

Apomorphine is administered via subcutaneous injections, either intermittently or as a continuous infusion. The common injection sites are the abdomen and outer thighs. The average bolus dose is 1–5 mg and the average daily dose is 3–30 mg. The individual dose should not exceed 10 mg and the total daily dose should not exceed 100 mg. If more than 10 injections are needed in a day, apomorphine should be administered via a continuous subcutaneous infusion pump. The infusion range is 1–4 mg/h (15–60 μ g/kg/h). Apomorphine infusion at 4 mg/h is equivalent to 600 mg of levodopa. When initiating the test dose of apomorphine, it is preferable to do this under observation in a hospital with monitoring facilities. The delivery of apomorphine via intranasal, sublingual, and transdermal routes are currently under investigation. Apomorphine should not be given via an intravenous infusion because this can precipitate intravascular thrombosis [185].

The side effects of apomorphine include nausea, vomiting, dizziness, orthostatic hypotension, somnolence, dyskinesia, and hallucinations [186]. The nausea can be severe and requires pretreatment with an antiemetic for three days. Domperidone 20 mg three times daily or trimethobenzamide (Tigan) 300 mg three times daily is usually effective. The antiemetics should be continued for at least six weeks before tailing off the dose. Tolerance to nausea has often developed by then. Another common side effect with subcutaneous apomorphine injection is the development of indurations and nodules at the injection sites. Rotating the injection sites may help to reduce their occurrence. Nodules and indurations may be treated with low-frequency ultrasound. Hemolytic anemia has been reported in patients treated with apomorphine. In some countries, hematological studies are recommended at baseline and every 6–12 months thereafter.

17.5.1.4 Monoamine Oxidase Type B (MAO_B) Inhibitors. MAO is an intracellular enzyme that is distributed widely in the body and plays a crucial role in the catabolism of catecholamines (including dopamine) and serotonin. There are two types of MAO, type A and type B. The predominant subtype in the brain is MAO_B. It metabolizes dopamine to DOPAC, which is further metabolized to HVA via COMT. It also converts the COMT metabolite 3-methyl-tyramine to HVA (Fig 17.1).

17.5.1.4.1 Selegiline. Selegiline is a selective irreversible MAO_B inhibitor. By inhibiting the oxidative deamination of dopamine in the brain, it helps to prolong dopaminergic transmission. It has been suggested to have a neuroprotective effect [187, 188], but the evidence is unconvincing as selegiline has been shown to have mild symptomatic effect and a limited duration of efficacy [189].

Selegiline is available as a 5-mg tablet, and the recommended daily dose is 5–10 mg. It is absorbed from the gastrointestinal tract and undergoes extensive metabolism to major plasma metabolites such as *N*-desmethylselegiline, *L*-amphetamine, and *L*-methamphetamine [41]. *N*-Desmethylselegiline has MAO_B inhibiting activity. Selegiline causes irreversible MAO_B inhibition, and the clinical effects may last longer than its plasma elimination half-life of 0.1–2 h. Food increases the bioavailability of selegiline by 3 to 4 times. The selectivity for MAO_B inhibition is dose dependent; at higher doses, MAO_A inhibition is also present.

There have been reports of selegiline inducing atrial fibrillation [190] and causing increased mortality when used in combination with levodopa [191]. The mortality was greatest in the third and fourth years of follow-up. These patients were more likely to have cognitive deficits and frequent falls [192]. However, a separate meta-analysis of five clinical trials involving selegiline did not demonstrate any increase in mortality, whether or not levodopa was given [193]. Nevertheless, the combination may increase dopaminergic side effects such as hallucinations and dyskinesia. Selegiline should be used with caution in patients with cognitive deficits and postural hypotension.

The major side effect of selegiline is insomnia [194]. Other side effects include nausea, dizziness, abdominal pain, confusion, hallucinations, dry mouth, headache, anxiety, and vivid dreams and may be exacerbations of levodopa therapy. Because of its effects on sleep, it is advisable to take the dose at breakfast and lunch only. The recommended daily dose should not be exceeded, because MAO_A inhibition occurs at higher doses. Inhibition of intestinal MAO_A will lead to a hypertensive crisis when

exogenous amines (such as those found in cheese, red wine, and certain cough mixtures) are ingested—the “cheese effect.”

Selegiline may cause adverse reactions when taken with certain medications [194]. The combination of selegiline and meperidine can cause stupor, muscular rigidity, severe agitation, elevated temperature, and in severe cases death. There have been reports of hyperpyrexia and death with the combination of selegiline and tricyclic antidepressants. This combination can lead to agitation, restlessness, tremor, behavioral changes, hypertension, muscular rigidity, diaphoresis, seizures, syncope, and asystole. The combination of selegiline and selective serotonin reuptake inhibitors (SSRIs) may also lead to the serotonin syndrome—hyperpyrexia, rigidity, myoclonus, autonomic instability, agitation, delirium, coma, and death. Selegiline should be discontinued at least for 14 days before starting tricyclic antidepressants or SSRIs.

17.5.1.4.2 Rasagiline. Rasagiline is a second-generation irreversible MAO_B inhibitor that is 3–15 times more potent than selegiline [195]. It is a propargylamine compound that does not give rise to amphetamine-like metabolites, an advantage over selegiline [196]. In vitro and in vivo studies in animals have suggested symptomatic and neuroprotective effects of this drug [197–199], but this is unproven in humans. The median time to peak plasma concentration is 30 min [200], and its special pharmacokinetic and pharmacodynamic properties allow it to be given only once daily. Rasagiline is a new drug awaiting Food and Drug Administration (FDA) approval in United States. The side effects reported so far include headache, dizziness, insomnia, and nausea [200, 201].

17.5.1.5 COMT Inhibitors. Levodopa is metabolized by peripheral COMT to 3-OMD (Fig. 17.1). Inhibition of peripheral COMT will prolong the plasma elimination half-life of levodopa, and this ultimately translates to a longer clinical levodopa response. COMT inhibitors may be useful in patients who are experiencing end-of-dose “wearing-off” symptoms. There are two reversible COMT inhibitors: tolcapone and entacapone. COMT inhibitors double the bioavailability of levodopa. The peak plasma concentration of levodopa and the time to peak plasma concentration are relatively unaffected [41].

17.5.1.5.1 Tolcapone. Tolcapone was introduced in 1998. Soon after, there were three cases of fatal hepatotoxicity reported [202, 203]. As a result, it was withdrawn in most countries and may be prescribed only under special circumstances in the United States and Canada. The current recommended daily dose is 100 mg three times daily, to be given only as an adjunctive therapy to levodopa. It is reserved for patients who are experiencing motor fluctuations and not responding satisfactorily to other adjunctive treatments. Hepatic function should be tested prior to starting tolcapone and then every two weeks for the first year followed by every four weeks for the next six months and then according to local regulatory requirements.

Tolcapone is absorbed rapidly from the gastrointestinal tract and reaches peak plasma concentration within 2 h [41]. It is metabolized in the liver. The major metabolic pathway is glucuronidation to inactive glucuronide. It is also methylated by COMT to 3-*O*-methyl-tolcapone and oxidized by the cytochrome P450 system. Its plasma elimination half-life is approximately 2–3 h. At higher doses, tolcapone may cross the blood–brain barrier and inhibits central COMT [204, 205].

The side effects of tolcapone are mostly related to enhanced dopaminergic effects. Dyskinesia, nausea, sleep disturbances, orthostatic hypotension, hallucinations, and confusion are the common side effects [206]. Another side effect is severe diarrhea necessitating withdrawal of the drug. It occurs in 16–18% of patients treated with tolcapone [206]. It usually develops within 6–12 weeks of tolcapone treatment but may present as early as 2 weeks and as late as many months later.

17.5.1.5.2 Entacapone. Entacapone has been available since 1999. Unlike tolcapone, it does not inhibit central COMT. It is absorbed from the gastrointestinal tract and reaches peak plasma concentration within 1 h. It is metabolized mainly in the liver via glucuronidation. The plasma elimination half-life is approximately 2 h [206]. Because its pharmacokinetic profile is similar to that of levodopa, the recommended dosing schedule is one 200-mg tablet of entacapone, to be taken together with individual levodopa doses, up to a maximum of eight times daily.

The side effects of entacapone are mainly enhanced dopaminergic effects such as dyskinesia, nausea, dizziness, orthostatic hypotension, and hallucinations [206]. Nondopaminergic side effects are diarrhea, abdominal pain, and urine discoloration. So far, there have been three possible cases of entacapone-induced hepatic dysfunction [207]. Currently, there is no requirement to monitor hepatic enzymes during entacapone treatment. It should be used with caution in patients with hepatic impairment.

17.5.1.5.3 Stalevo. It is thought that continuous dopaminergic stimulation may reduce dyskinesia and motor fluctuations [208]. This has led to the introduction of a “3-in-1” tablet that contains levodopa, carbidopa, and entacapone (Stalevo) [40]. The pharmacology of Stalevo is equivalent to adding a 200-mg entacapone tablet to a standard Sinemet dose with a levodopa–carbidopa ratio of 4:1.

17.5.2 Nondopaminomimetic Agents

17.5.2.1 Anticholinergic Drugs. Drugs with anticholinergic properties were the first effective treatment for Parkinson's disease (long before the role of acetylcholine was discovered). Their antiparkinsonian effects are mild but their side effects are significant. With the introduction of levodopa therapy and dopamine agonists, anticholinergic drugs are less commonly used.

Trihexyphenidyl or benzhexol and bengtropine are the more common anticholinergic agents used in Parkinson's disease. They act on the G-protein-linked muscarinic receptors. The exact mechanisms of action of these agents in Parkinson's disease are not known. In addition to its antimuscarinic action, bengtropine blocks the reuptake of dopamine at the dopamine transporter level [209], thereby prolonging the effects of dopamine at the synaptic cleft. Anticholinergic drugs have a particular role in relieving tremor [210].

Trihexyphenidyl is available in 2- and 5-mg tablets. The average dose range is between 4 and 8 mg daily. Younger patients tend to tolerate higher doses better than older patients. The time to peak plasma concentration is 2–3 h. The duration of action ranges from 1 to 12 h [211].

Antimuscarinic side effects include dry eyes, dry mouth, urinary retention, constipation, confusion, hallucinations, and blurred vision [211]. Elderly patients are particularly prone to these side effects. Other side effects include tachycardia,

impaired sweating, gastrointestinal obstruction, and megacolon. Anticholinergic agents may also precipitate acute angle glaucoma.

17.5.2.2 *Antiglutamate Agents*

17.5.2.2.1 Amantadine. Amantadine was initially marketed as a treatment for influenza but was discovered by chance [212] to have beneficial effects in Parkinson's disease. It has been used either as a monotherapy [213] or as an adjunctive treatment to levodopa [214] and anticholinergic agents [215].

There are multiple proposed sites for the antiparkinsonian action of amantadine. It is thought to induce release of dopamine at the presynaptic level [216], to block the reuptake of dopamine [217], to act on postsynaptic dopamine receptors [218], and to increase expression of striatal D₂ receptors [219]. In addition, it has anticholinergic properties [220] and antiglutamate effects [221, 222] through blockade of *N*-methyl-D-aspartate (NMDA) receptors.

Interest in amantadine has resurfaced recently with reports of its antidyskinetic effects [223–227]. Chase and Oh [228] suggested that the antidyskinetic properties might be related to glutamate antagonism. The pulsatile action on dopamine receptors of the striatal medium spiny neurons causes abnormal phosphorylation of NMDA receptors, thus perhaps leading to plastic changes and development of motor complications [229]. The antidyskinetic effects of amantadine seldom last for more than a year [227].

Amantadine is available as a 100-mg capsule and syrup. The recommended dose range is 100–200 mg daily in two divided doses. The time to peak plasma concentration is 1–12 h, and the plasma elimination half-life may last more than a day [41]. Amantadine is excreted unchanged in the urine. It should be used with caution in patients with renal impairment, urinary tract infection, and dehydration.

The side effects of amantadine include nausea, dizziness, insomnia, dry mouth, constipation, leg edema, livedo reticularis, nervousness, depression, confusion, and hallucinations [213, 230, 231]. Fatal toxicity has been reported from suicidal overdose [232, 233]. Abrupt withdrawal or dose reduction can precipitate a rebound psychosis or neuroleptic malignant syndrome [234]. Amantadine should be tapered and stopped over a period of two weeks.

17.6 TREATMENT OF MOTOR COMPLICATIONS

Approximately 20–50% of patients treated with levodopa for three to five years will develop motor complications [44, 66–72]. It has been reported that patients on long-term levodopa treatment have a higher peak plasma levodopa concentration, a shortened time to peak plasma concentration, and a shortened elimination half-life [235]. The therapeutic window is narrowed, and the dose–response curve becomes steeper [236, 237].

The pathophysiology of motor complications is not entirely clear. Possible mechanisms include a reduced dopamine storage capacity [73], alterations in postsynaptic receptor sensitivity [238, 239], differential involvement of dopamine receptors [240, 241], and chronic intermittent dopaminergic stimulation that leads to downstream receptor changes [74, 75]. Table 17.3 lists the different types of motor complications and their suggested treatment.

TABLE 17.3 Treatment of Motor Fluctuations and Dyskinesia

Motor Complications		Treatment
Motor fluctuations	Wearing off	Increase levodopa dose
		Increase dose frequency
		Switch to slow-release levodopa
		Add dopamine agonist
		Add COMT inhibitor
	Sudden 'off'	Subcutaneous apomorphine injection
	Delayed 'on'	Dietary modification
		If taking slow-release levodopa, add/ switch to standard levodopa
		Dispersible levodopa
		Subcutaneous apomorphine injection
Dyskinesia	Dose failure	Dietary modification
		Liquid levodopa
		Subcutaneous apomorphine injection
	Peak-dose dyskinesia	Lower levodopa dose
		Add dopamine agonist
	Diphasic dyskinesia	Add amantadine
		Add dopamine agonist
		Add amantadine
	Painful or 'off' dystonia	Increase levodopa dose/frequency
		Increase levodopa dose
	Yo-yo-ing	Add dopamine agonist
		Liquid levodopa
		Continuous subcutaneous apomorphine injection

17.6.1 Wearing Off

This is the most common and usually earliest motor fluctuation encountered. The clinical response following a single dose of levodopa is not sustained for more than 4 h, and patients experience a return of parkinsonian symptoms before the next dose is due. This problem can be overcome by increasing the levodopa doses, shortening the dosing intervals, switching from immediate-release levodopa to the slow-release formulations, adding a COMT inhibitor, or adding a dopamine agonist. With these measures, the plasma levodopa concentration is maintained at the therapeutic level for a longer period. However, another complication may arise such as peak-dose dyskinesia. Adding a dopamine agonist is less likely to precipitate dyskinesias.

17.6.2 Sudden "Off"

Sudden off is more difficult to treat because it is not related to the dosing schedule. This phenomenon does not respond well to existing oral antiparkinson medications. Sub-

cutaneous apomorphine injections may be the only effective treatment for the off periods.

17.6.3 Delayed “On”

The onset of clinical response following a single dose of levodopa may be delayed. The underlying mechanism is not clear but may be due to inadequate plasma levels of levodopa. The absorption of levodopa may be increased by redistributing dietary protein, adding a booster dose of regular Sinemet, or switching Sinemet CR to regular Sinemet. Dispersible levodopa or subcutaneous apomorphine injections may be added to provide a more rapid onset of clinical response.

17.6.4 Dose Failure

Sometimes there may be no response following a single dose of levodopa. This may be due to fluctuations in the absorption of levodopa or changes in the dopamine receptor sensitivity. Dose failure is most often unpredictable. The off period may be helped by liquid levodopa or subcutaneous apomorphine injections.

17.6.5 Peak-Dose Dyskinesia

Peak-dose dyskinesia is a common feature of motor complications. As the name implies, dyskinesia occurs at the peak levodopa plasma level. It usually takes the form of chorea, often with dystonia [242]. The upper body is affected more than the lower limbs.

Lowering the levodopa doses will often help to reduce the dyskinesia, but this may also aggravate the parkinsonian symptoms. To overcome this, a dopamine agonist may be added. Dopamine agonists have direct dopaminomimetic effects, and their duration of action is longer than levodopa. They allow a lower dose of levodopa to be maintained without compromising the antiparkinsonian effects. Another option is to add amantadine.

17.6.6 Diphasic Dyskinesia

Dyskinesia may also occur during the rising and falling phase of plasma levodopa level [242]. Dystonia is a more common feature in this type of dyskinesia. The lower limbs are often affected.

Treatment of diphasic dyskinesia is difficult. It may be helpful to add a dopamine agonist or amantadine.

17.6.7 Painful Dystonia

Patients may complain of painful dystonia, especially in the morning before the first dose of treatment. This is an off phenomenon, related to the lower plasma levels of levodopa. Management is to increase the dopaminergic stimulation by either increasing levodopa or adding dopamine agonists.

17.6.8 Yo-yo-ing

Patients may fluctuate between the off states and dyskinesia like a yo-yo. It is difficult to manage this type of motor complication because treating one problem will lead to another. A combination of dopamine agonists, amantadine, or COMT inhibitors with levodopa may be effective. Patients often prefer to be in the on state with dyskinesia rather than being off. In advanced cases, liquid levodopa or continuous subcutaneous apomorphine injections may be necessary.

17.7 TREATMENT OF NONMOTOR SYMPTOMS

The management of the nonmotor symptoms of Parkinson's disease is difficult. The symptoms are diverse and generally require multiple approaches. Some of these symptoms are inherent to the disease while others may be a result of treatment. Agents used to treat the nonmotor symptoms (especially complications of treatment) may worsen the motor manifestations of Parkinson's disease.

17.7.1 Nausea and Vomiting

Nausea and vomiting are common side effects of dopaminomimetic agents. These drugs act on the chemoreceptor trigger zone which is located in the brain stem but outside the blood-brain barrier. Simple measures like taking the medication with food or increasing the dosage slowly can often help to reduce these side effects. Domperidone is very helpful—it is a dopamine receptor antagonist that does not cross the blood-brain barrier. In the United States, where domperidone is not available, trimethobenzamide (Tigan) may be used. These drugs do not affect the motor symptoms of Parkinson's disease, unlike other antiemetics such as metoclopramide [243].

The usual dose of domperidone is 10–20 mg three to four times daily, to be taken half an hour before dopaminomimetic agents. The usual dose of trimethobenzamide is 300 mg three times daily. Domperidone should not be used in women with a history of unilateral breast cancer.

17.7.2 Orthostatic Hypotension

Orthostatic hypotension may be a late feature of Parkinson's disease, but more often it is a treatment complication [48]. Stimulation of the cardiovascular dopamine receptors reduces the peripheral vascular resistance, and this leads to hypotension. Titrating the dopaminomimetic agents slowly may help to reduce this side effect. Increasing the salt and fluid intake may also help to bring up the blood pressure. Fludrocortisone or midodrine is usually beneficial. Fludrocortisone is a potent synthetic mineralocorticoid. Midodrine is a peripheral acting α_1 -adrenergic agonist. Both agents are effective in raising the blood pressure and can be given in Parkinson's disease. The usual dose range of fludrocortisone is 0.1 mg once or twice daily. The usual daily dose range of midodrine is between 2.5 and 20 mg.

TABLE 17.4 Similarities between Features of Parkinson's Disease and Depression

Parkinson's Disease	Depression
Loss of spontaneous facial expression	Loss of spontaneous facial expression
Motor and mental slowing due to bradykinesia and bradyphrenia	Motor and mental slowing due to psychomotor retardation
Inability to pursue hobbies and interests due to physical symptoms	Inability to pursue hobbies and interests due to apathy
Decreased sexual activity due to immobility and loss of libido	Decreased sexual activity due to loss of libido and drug therapy
Impaired cognitive-intellectual functions due to dementia	Impaired cognitive-intellectual functions due to pseudodementia
Slowed executive function	Slowed executive function
Lack of concentration	Lack of concentration
Agitation, anxiety	Agitation, anxiety
Hallucinations, delusions, paranoia	Hallucinations, delusions, paranoia
Sleep disturbance	Sleep disturbance
Constipation	Constipation

17.7.3 Depression and Anxiety

Depression affects 40–50% of patients with Parkinson's disease [244]. Sometimes depression may occur only during the off phase of Parkinson's disease.

It is important to ask about depression in Parkinson's disease because treatment has a major impact. The symptoms of depression may often mimic the features of Parkinson's disease, such as hypomimia, hypophonia, psychomotor retardation, and fatigue (Table 17.4). Depression in Parkinson's disease may occur anytime, even prior to the onset of motor symptoms. It usually presents as a lack of motivation and is often associated with anxiety and panic attacks [245]. Self-blame, guilt, and suicide are rare [246, 247].

Treatment of depression in Parkinson's disease is no different from treatment in depressed patients without Parkinson's disease although older Parkinson's disease patients generally do not require extensive psychotherapy; however, young onset patients may require intensive therapy. Antidepressants and in severe cases electroconvulsive therapy may be used. Tricyclic antidepressants, SSRIs, and atypical antidepressants are all effective. Examples of tricyclic antidepressants include amitriptyline and nortriptyline. Examples of SSRIs include fluoxetine (Prozac), sertraline (Zoloft), paroxetine (Paxil), and fluvoxamine (Luvox). Venlafaxine (Effexor) is a useful atypical antidepressant that inhibits the reuptake of serotonin and noradrenaline. Mirtazapine (Remeron) enhances central noradrenergic and serotonergic activity.

Tricyclic antidepressants have anticholinergic, antihistaminergic, antiadrenergic, and antiserotonergic effects. They are sedating and may be useful in patients with coexisting anxiety and insomnia. However, the anticholinergic side effects generally limit their use, especially in elderly patients. Tricyclic antidepressants may worsen confusion, orthostatic hypotension, and somnolence in those patients who are already having these symptoms. They may also cause cardiac arrhythmias. The

combination of tricyclic antidepressants and MAO inhibitors has been reported to cause hyperpyrexia, mental changes, muscular rigidity, syncope, and death [194].

The SSRIs have a better safety profile than the tricyclic antidepressants. They have fewer anticholinergic side effects and lower incidences of cardiac arrhythmia. Fluoxetine has a central activating effect and may be useful in patients who are apathetic. However, it is unhelpful if the patient is having agitation and mania. Fluvoxamine is sedating and is useful in patients who have insomnia. SSRIs should not be given together with MAO inhibitors because this may precipitate a serotonin syndrome [194]. In addition, there have been reports of SSRIs worsening the motor symptoms of Parkinson's disease [248, 249]. Fortunately, such occurrences are rare.

In situations where depression occurs only during the off phase of levodopa treatment, improvement can be achieved by optimizing the levodopa therapy. Pramipexole has been claimed to have antidepressant properties [159, 160] and it may be considered in patients with Parkinson's disease and depression.

Anxiety is particularly common in Parkinson's disease [246]. Anxiety often exacerbates the motor symptoms of Parkinson's disease, in particular tremor. Treatment options include counseling, short-acting anxiolytics, antidepressants, and optimization of dopaminomimetic therapy. The commonly used anxiolytics are lorazepam, alprazolam, and buspirone, an antidepressant with anxiolytic properties. These drugs may increase confusion in patients who are already cognitively impaired.

17.7.4 Dementia

Dementia is usually a late complication, affecting 20–40% of patients with Parkinson's disease [250–252]. The risk factors include age, disease duration, and disease severity. Associated symptoms include mood disturbances, hallucinations, psychosis, and confusion. While dementia is common in advanced Parkinson's disease, it can also occur in mild Parkinson's disease—in fact, it can even precede Parkinson's disease. Over the last decade there has been increasing interest in the dementia of Parkinson's disease and the blurring of distinctive pathological features of Parkinson's disease and Alzheimer's disease. A number of terms have been coined for the clinical and pathological findings associated with dementia in Parkinson's disease. These include *diffuse Lewy body disease* [253], *cortical Lewy body disease* [254], *dementia with Lewy bodies* [255], and *Parkinson's disease dementia* [256]. Debates continue on the criteria for separating different forms of dementia in Parkinson's disease, but some neurologists are becoming sceptical about our ability to do anything more than recognize an association between Parkinson's disease and Alzheimer's disease and perhaps a tendency for the progression of either to lead to some convergence if patients live long enough [257].

There have been favorable reports of treating dementia with cholinesterase inhibitors [258, 259]. In particular, these drugs do not appear to aggravate the motor features of parkinsonism and may be useful in treating dementia in Parkinson's disease. So far, there have been trials involving the use of donepezil, rivastigmine, and galantamine in Parkinson's disease. Donepezil may improve cognitive deficits [260], psychosis [261], and hallucinations [262]. Similarly, there have been reports of rivastigmine and galantamine improving cognitive deficits [263, 264]. However, worsening of motor features has been documented in some patients [260, 262, 263].

17.7.5 Psychosis and Hallucinations

Psychosis and hallucinations are dose-dependent complications of antiparkinson medications when used in patients with Parkinson's disease. They may also be features of advanced Parkinson's disease, especially in those with cognitive deficits. Management involves simplifying the treatment regimes as well as using atypical neuroleptics such as clozapine or quetiapine.

Drugs with the least antiparkinsonian effects but with substantial neuropsychiatric complications are often withdrawn first. These include MAO_B inhibitors, anticholinergic agents, and amantadine. The next group of drugs to be withdrawn, or to have their dosages reduced include COMT inhibitors and dopamine agonists. Finally, patients are maintained on the lowest possible doses of levodopa. However, many patients with advanced Parkinson's disease cannot tolerate a major reduction in dopaminomimetic agents. In such situations, atypical neuroleptics may be used.

Clozapine was the first atypical antipsychotic agent marketed and is still the most effective. It has high affinity for D₄ receptors, with less binding to the striatal dopamine receptors [265]. The usual dosage is between 25 and 100 mg daily in one to two divided doses; however, in the frail elderly patient 12.5–25 mg daily may be sufficient. Clozapine can cause life-threatening idiosyncratic agranulocytosis in 1–2% of patients. Baseline and biweekly white cell count monitoring is required. Patients should not be started on clozapine if the white cell count is below 3500 per cubic millimeter. The agranulocytosis is reversible if the drug is withdrawn within one to two weeks. Besides agranulocytosis, clozapine may also cause seizures, myocarditis, orthostatic hypotension, and sedation [266, 267]. It is metabolized by the cytochrome P450 enzymes and may be subject to drug interactions.

Quetiapine is a new atypical antipsychotic agent with D₂ receptor binding profile similar to clozapine [268]. It does not cause agranulocytosis, and regular blood count monitoring is not required. The usual prescribed dose is 25–50 mg daily. Quetiapine may cause orthostatic hypotension and drowsiness and is preferably given at night. Quetiapine has minimal effects on the motor symptoms of Parkinson's disease [269].

17.7.6 Other Nonmotor Symptoms

Constipation is a frequent complaint in Parkinson's disease [270, 271]. Possible causes include parasympathetic involvement, immobility, reduced fluid and fiber intake, and side effects of medications such as anticholinergic agents and codeine-based compounds. While most patients will require laxatives during the course of the disease, this should be balanced against the fact that Parkinson's disease patients with constipation are at increased risk of developing intestinal obstruction, paralytic ileus, volvulus, and gut perforation [270]. Patients with advanced disease often have difficulty maintaining adequate exercise or drinking enough fluids. They may also choke on bran that swells up. A good bowel management program is therefore essential [270].

Urinary symptoms such as frequency, urgency, and nocturia are also common complaints in Parkinson's disease [272, 273]. In male patients, it may be difficult to differentiate these symptoms from prostatic hypertrophy. Urodynamic studies may help. Urinary incontinence is not an early feature of Parkinson's disease but it is in multiple-system atrophy [274]. Detrusor hyperactivity may be managed by anticholinergic agents such as oxybutynin (5–10 mg at night). Oxybutynin, however, may

cross the blood–brain barrier [275] and cause neuropsychiatric side effects [276, 277]. It should be used with caution in patients with cognitive deficits. For male patients a condom catheter may be useful at night. Both men and women should consider using incontinence pads during the day [270].

Sleep disturbances may occur for a number of reasons [172, 278]. Patients may have fragmented sleep because of stiffness, nocturia, or restless leg syndrome. Depression, vivid dreams, or nightmares may also affect the quality of sleep. Sometimes patients have motor and behavioral symptoms while asleep (REM sleep behavior disorders). Interrupted sleep at night often leads to excessive daytime sleepiness. Certain dopaminomimetic agents, such as pramipexole and ropinirole, may cause daytime hypersomnolence.

Treatment depends on the underlying cause of the sleep disturbances. Clonazepam may reduce REM sleep behavior disorders [279]. Modafinil may be used to treat excessive daytime sleepiness [280].

Pain, numbness, and paresthesia have been reported in up to 50% of patients with Parkinson's disease [281, 282]. These symptoms may respond to dopaminomimetic agents, especially if they are related to stiffness and off dystonia.

17.8 NEUROPROTECTIVE THERAPY

It is not clear what causes cell death in Parkinson's disease. Various mechanisms have been postulated, and these include oxidative stress, mitochondrial dysfunction, cellular excitotoxicity, neuroinflammation, dysfunctional protein degradation, and apoptosis [283–287]. Drugs inhibiting one or more of these processes may potentially be neuroprotective.

The DATATOP (Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism) study [187] was the first clinical trial performed to evaluate neuroprotection in Parkinson's disease. Selegiline, also known as deprenyl, has been shown to delay levodopa treatment by nine months [188], and this was initially thought to represent neuroprotection secondary to its antioxidative properties [288]. This view was soon questioned [189]. Selegiline has mild symptomatic benefits, and the MAO_B inhibition can last for a long time [289]. Moreover, subsequent studies did not show a significant difference in levodopa-induced complications between treatment groups [290, 291]. It is therefore unlikely that selegiline is neuroprotective.

It has also been claimed that dopamine agonists have antioxidative effects. In addition, pramipexole may affect mitochondrial membrane potential and inhibit apoptosis [292]. In the CALM-PD study [95], the effect of pramipexole on disease progression was compared to levodopa, and ¹²³I-βCIT SPECT (single-photon emission computerized tomography) was used as a marker of dopamine transporter density. In the REAL-PET study [97], ropinirole was compared to levodopa in de novo Parkinson's disease, and neuroimaging was performed with ¹⁸F-dopa positron emission tomography (PET). Both studies suggested a slower rate of disease progression with dopamine agonists, based on neuroimaging findings. The clinical observations, however, showed a better outcome with the levodopa treatment group. There have been several unresolved issues with these studies, including the possible pharmacological influences on the neuroimaging ligands [162, 163].

Glutamate is an excitatory neurotransmitter and may induce excitotoxicity. In animal models of parkinsonism, the use of NMDA receptor antagonists have been shown to protect dopamine neurons from glutamate-mediated toxicity [293, 294]. However, clinical trials have failed to show any neuroprotective effects of riluzole, a NMDA receptor antagonist [295].

Coenzyme Q₁₀ is the electron acceptor for mitochondrial complexes I and II. Studies have shown a reduced complex I activity and coenzyme Q₁₀ levels in Parkinson's disease [296, 297]. In a pilot clinical trial, patients treated with high doses of coenzyme Q₁₀ (1200 mg daily) have a slower rate of decline in their UPDRS scores [298]. The benefits, however, were in the subjective "activities of daily living" scores rather than the objective motor scores. The requirement for dopaminomimetic therapy was also not delayed.

Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of dopamine neurons [299]. When injected into MPTP-treated monkeys, there was improvement in parkinsonian features [300]. A phase 1 clinical trial with direct GDNF infusion into the putamen of Parkinson's disease patients showed not only clinical improvement with tolerable side effects but also an increase in ¹⁸F-dopa uptake on PET [9]. However, the clinical benefits were not reproducible in a subsequent multicenter, randomized, double-blind, placebo-controlled trial.

Other agents that have been studied include caffeine (adenosine antagonism), nicotine (antioxidation or anti-excitotoxicity), creatine (mitochondrial stabilizer), and minocycline (anti-inflammation or antiapoptosis). The preclinical evidence looks promising for some, and in 2003, twelve agents were selected by CINAPS (Committee to Identify Neuroprotective Agents in Parkinson's) for further studies [301]: caffeine, coenzyme Q₁₀, creatine, estrogen (17- β -estradiol), GM-1 ganglioside, minocycline, nicotine, neuroimmunophilin (GPI-1485), rasagiline, selegiline, pramipexole, and ropinirole.

Unfortunately, the accumulated evidence, at present, does not justify optimism in the search for neuroprotection. We have many theories but no facts that allow a careful observer to conclude that any drug confers either protection or rescue from the neurodegenerative mechanism that kills cells in Parkinson's disease.

17.9 TREATMENT IN THE NEAR FUTURE

Apomorphine has recently been approved for use in the United States to treat Parkinson's disease. Very soon, rasagiline, a second-generation MAO_B inhibitor, will join the league. Other drugs in clinical trial include the ethyl ester preparation of levodopa, new formulations of dopamine agonists, and a variety of non-dopaminomimetic agents such as adenosine A_{2A} receptor antagonists, monoamine reuptake inhibitors, and agents that interact with the serotonergic system.

Transdermal preparation of a new dopamine agonist, rotigotine, will soon be available. Rotigotine is a nonergot, selective D₂ dopamine agonist. It is delivered through a silicone-based transdermal patch that releases drug throughout a 24-h period. This system provides a noninvasive method of delivering continuous dopaminergic stimulation. Preliminary studies have shown that it is effective in treating early Parkinson's disease [302]. It may also reduce motor fluctuations in advanced disease [303]. The side effects include nausea, dizziness, and application site reactions which are mild and well tolerated. Hallucinations are rarely encountered. Another skin patch that is currently undergoing development is transdermal lisuride [147].

Adenosine A_{2A} receptors are concentrated mainly in the striatum, nucleus accumbens, and olfactory tubercle [304]. They are intricately related to the striatal GABAergic medium spiny neurons and the cholinergic aspiny neurons. Stimulation of adenosine A_{2A} receptors modulates the release of neurotransmitters, such as acetylcholine, GABA, glutamate, and dopamine [305]. It is believed that adenosine suppresses GABAergic feedback inhibition within the striatum. Together with nigrostriatal dopaminergic and cortical glutamatergic inputs, they modulate overall functioning of the indirect pathway of the basal ganglia motor loop. In Parkinson's disease, there is excessive activation of the indirect pathway. It is believed that adenosine A_{2A} receptor antagonists will increase GABAergic feedback inhibition within the striatum, thus leading to a reduced output from the indirect pathway. This ultimately translates to increased locomotive activity, as seen in MPTP-treated monkeys given oral istradefylline, an A_{2A} receptor antagonist [306]. Initial clinical trial on patients with advanced Parkinson's disease showed that istradefylline had no effects on parkinsonian symptoms when given on its own, but when added to low-dose levodopa, it potentiated the antiparkinsonian effects without exacerbating dyskinesia [307]. Other preclinical studies have shown adenosine modulating functions such as sleep [308] and mood disturbances [309], suggesting that adenosine A_{2A} receptor antagonists may be used as arousal agents or antidepressants. There were also suggestions that adenosine A_{2A} receptor antagonists may have potential neuroprotective functions [310]. Multicenter, randomized controlled trials studying the effects of istradefylline in advanced Parkinson's disease with motor fluctuations are currently in progress.

17.10 CONCLUSION

Our understanding of the pharmacology and practice of treatment for Parkinson's disease has evolved explosively since the discovery of dopamine as a neurotransmitter and its depletion in Parkinson's disease. In many respects the story is an outstanding example of the rational application of laboratory observations into clinical practice. The advances in symptomatic treatment have been spectacular, although management of adverse reactions remains a challenge. There has been relative neglect of those aspects of Parkinson's disease that are not related to dopamine depletion. The burning issue, however, is the task of slowing the pathogenesis of Parkinson's disease. To achieve this, we need to understand the mechanism of cell death, and this is where the major thrust of research should be applied.

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18

PARKINSON'S DISEASE: GENETICS AND PATHOGENESIS

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18.1	Introduction	524
18.2	Monogenetic Parkinsonism	525
18.2.1	Causative Mutations	527
18.2.1.1	PARK1 and PARK4: <i>SNCA</i>	527
18.2.1.2	PARK2: <i>parkin</i>	529
18.2.1.3	PARK6: <i>PINK1</i>	532
18.2.1.4	PARK7: <i>DJ-1</i>	535
18.2.1.5	PARK8: <i>LRRK2</i>	536
18.2.2	Potential Causative Mutations	539
18.2.2.1	PARK5: <i>UCH-L1</i>	539
18.2.2.2	Mutations Outside of PARK Loci	540
18.2.3	Parkinsonism Loci	540
18.2.3.1	PARK3	541
18.2.3.2	PARK9	541
18.2.3.3	PARK10	541
18.2.3.4	PARK11	541
18.3	Genetics and IPD	542
18.3.1	Disease Modifiers: Age of Onset	542
18.3.2	Disease Risk	542
18.3.2.1	GBA Polymorphisms	543
18.3.2.2	Mitochondrial DNA	543
18.4	Environment and IPD	543
18.4.1	Lifestyle: Smoking, Caffeine, and Alcohol	544
18.4.2	Vitamins	544
18.4.3	Pesticides	545
18.5	Mitochondria and Oxidative Stress: One Working Theory of PD Pathogenesis	545
18.6	Conclusion	547
	Acknowledgments	547
	References	547

18.1 INTRODUCTION

The definition of idiopathic Parkinson's disease (IPD) remains clinical, with neuropathology as the gold standard to confirm diagnosis. However, a combination of medical genetics and careful neuropathology is slowly shifting definitions and treatment of IPD. The cardinal clinical signs defining IPD, rest tremor, rigidity, and bradykinesia, are related to nigrostriatal dysfunction and are the main targets of symptomatic therapy. In contrast, theories of pathophysiology must explain a wider range of neuronal involvement. Other brain stem regions, such as olfactory nucleus, show pathological changes before substantia nigra; cerebral cortex is affected in later stages of disease [1]. Disease features may reflect pathology in these other areas; for example, a systematic literature review suggests that about a quarter of IPD patients have dementia [2]. New treatments must address symptoms such as dementia, autonomic dysfunction, and sleep disorders that are unresponsive to dopaminergic agents.

The other enormous gap in IPD pharmacotherapeutics is the total lack of effective neuroprotective agents. Design of potential neuroprotective treatments is closely tied to the key debates on IPD definition and pathophysiology: Is IPD caused by one major mechanism or several? Are IPD and other parkinsonisms closely related or are the common pathways observed in neurodegenerative diseases late reactive effects instead of causal? Even experienced neurologists have a diagnostic accuracy for IPD of about 80% when compared to neuropathology [3]. The difficulty in distinguishing IPD from other syndromes comes from our gaps in understanding the full clinical spectrum of IPD and from lack of a clear, unified understanding of pathophysiology. Creation of effective therapeutics rests on our ability to break out of this circle by eventually clearly categorizing IPD and parkinsonisms by neurobiologically related causes.

Classification of IPD still rests on the observation of Lewy bodies and Lewy neurites on neuropathology [1]. Even this basic categorical premise is being challenged by work in medical genetics. Some genetic forms of parkinsonism lack Lewy bodies, while others have unique additional pathological characteristics. Familial disorders may perfectly mimic IPD or overlap with IPD but clearly differ in mean age of onset, progression, and extent of symptoms. Given this diversity, caution must be used in equating all genetic parkinsonisms with IPD until we change how Parkinson's disease (PD) is defined or understand basic neurobiological questions in PD, such as the creation of Lewy bodies (see [4] for a review). If Lewy bodies directly reflect a cause of PD, then they are a necessary feature of an etiological disease category, and parkinsonisms, including familial disease, that lack Lewy bodies are separate entities. If Lewy bodies are a late indicator of cell survival, it is possible that they will be absent in different phenotypes in the same pathophysiological group. For now, work on IPD pathophysiology struggles outside of a framework for diagnosing disease by etiological pathway, not clinical or pathological observation.

As classification of IPD versus familial or other parkinsonisms continues to shift, ongoing advances in understanding strictly inherited forms of PD are driving investigations of the pathophysiology of sporadic PD. Work on potential genetic modifiers of IPD risk, disease characteristics, and response to treatment is uncovering the connections between complex genetics and environmental factors underlying onset and progression of disease.

For a detailed discussion on the clinical pharmacology and therapy of PD see Chapter 17.

18.2 MONOGENETIC PARKINSONISM

Familial PD was reported as early as 1880 [5]. However, the enormous surge in interest in monogenetic parkinsonism in relationship to IPD is very recent. While unusual families with clear Mendelian inheritance patterns of PD have long been reported [6], risk of PD in relatives of patients with typical IPD (onset older than 60 years) is low, around two to three times that for relatives of controls [7–9]. A younger (under 50 years) age of onset in affecteds and presence of multiple affected relatives increase the potential hereditary bias for PD [8–10]. Although younger age of onset increased the potential genetic contribution to disease in twin studies [10], overall twin studies of IPD showed a low rate of concordance in both monozygotic and dizygotic twins, implying a minor role for genetic causes in IPD [10, 11]. Work was therefore invested in environmental or toxic factors in IPD, discussed in Section 18.4. However, recent studies of individual large kindreds have directly tied specific mutations to familial PD, reviving interest in genetic mutations. Understanding what role, if any, the associated genes and proteins play in IPD is a rapidly moving field.

Several inherited disorders share some phenotypic overlap with PD, including frontotemporal dementia (FTD17), X-linked dystonia/parkinsonism, spinal cerebellar atrophy (SCA) types 2 and 3, and dopa-responsive dystonia (DRD) [12]. How these entities are pathophysiologically related to PD, if at all, remains an open question. For simplicity, this chapter only discusses the assigned PARK loci linked to familial disease with significant overlap with IPD (Table 18.1) and a few other genetic changes potentially related to classic PD. Already one PARK locus, PARK9, has fallen out of use; as the associated clinical syndrome became better understood, it moved further away from IPD (see below). Whether any of the other clinical syndromes listed above will be added to the PARK list in the future or more PARK loci will “lose” their PD label awaits better understanding of PD and general neurodegenerative neurobiology.

In part because of this evolving understanding of genotype/phenotype correlations, PD genetic testing, either diagnostic or presymptomatic, remains difficult, with a high potential for unclear results [12, 13]. Genetic counseling and education for patients and families is crucial given the complexity of the field. A thorough understanding of current genotypes and clinical syndromes of inherited disease, including gaps in our knowledge of mutation rate and diversity, is a must for the practitioner.

There are now nine distinct PD-related genetic loci (excluding PARK9; PARK1 and PARK4 are tied to the same gene). Mutations in five genes unambiguously cause familial PD: *SNCA* (PARK1), *parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7), and *LRRK2* (PARK8). Mutation of a sixth gene in the PARK5 locus and in genes outside designated PARK loci has not been confirmed (discussed below). Experimental work on mutations and associated proteins has outlined overlapping theories of disease pathogenesis, including abnormal protein aggregation, proteasome dysfunction, oxidative stress, and mitochondrial dysfunction. Common challenges to moving forward from specific mutation identifications are that the associated

TABLE 18.1 The PARK loci. Specific genetic mutations within PARK3, PARK10 and PARK11 have not been identified. Genes listed contain mutations clearly linked to disease; genomic multiplications of *SNCA* (originally PARK4) also cause disease. A mutation in the PARK5 gene *UCH-L1* is not confirmed as causal, see text. The normal functions of most PD-associated mutation proteins products of are unknown. Some speculative functions are listed; for example, DJ-1 may protect cells from oxidative stress. General phenotype information is listed. As more cases are identified for each locus, some phenotypes are expanding to include a wide range of clinical and pathologic features. This is especially true for PARK8 and PARK2. PARK9 is not currently considered a PD locus; the associated disease has many features outside of parkinsonism, and the genetic mutation remains unknown. AD, autosomal dominant; AR, autosomal recessive.

Locus	Inheritance	Chromosome	Gene	Protein Name and Function	Phenotype
PARK1/ PARK4	AD	4q21	<i>SNCA</i> (mutation/ multiplication)	α -Synuclein synaptic regulation?	Mutations: range from typical PD to early-onset PD with other features to Lewy body dementia (LBD). Genomic triplication: early onset, rapid course, dementia, widespread disease for duplications with milder disease
PARK2	AR	6q25-27	<i>parkin</i>	Parkin E3 ligase	Variable age of onset, most early onset, slow progression, dystonia, relief with sleep, some lack Lewy bodies
PARK3	AD	2p13	—	—	Parkinsonism and dementia
PARK6	AR	1p35–36	<i>PINK1</i>	PINK1 protein kinase	Early onset, usually otherwise typical PD, slow progression
PARK7	AR	1p36	<i>DJ-1</i>	DJ-1 oxidative stress?	Early onset PD, can have behavioral changes, postural tremor, dystonia, slow progression
PARK8	AD	12p11.2–13.1	<i>LRRK2</i>	Dardarin MAPKKK?	Most late-onset PD, some cases include wide range of features, some lack Lewy bodies
PARK10	AD	1p32	—	—	Large population screen of typical PD
PARK11	AD	2q36–37	—	—	Large population screen of typical PD

proteins are often ubiquitously expressed, and their normal functions are unknown or ill defined.

Autosomal-dominant (AD) disease currently includes causative mutations associated with PARK1/PARK4 and PARK8. A common theme in AD PD is more severe, younger onset, and more heterogeneous phenotypes compared to IPD. While work on the associated genes and proteins may be directly relevant to IPD pathogenesis and therapeutics, the phenotypes in monogenetic AD PD stretch definitions of the disease. New loci (PARK10 and PARK11) have been defined using AD inheritance models and IPD definitions of disease in screens of very large populations. Whether this method yields distinct mutations associated with classic PD syndromes remains to be seen.

Autosomal-recessive (AR) disease currently includes PARK2, PARK6, and PARK7. It is tempting to look for AR mutations in IPD cases, because family history may be scarce, especially in late-onset forms; however, genetic testing becomes more informative with early-onset disease and with good characterization of the exact phenotypes involved [12, 13]. The importance of single heterozygous (hemizygous) mutations in disease risk is still unclear. This will become a key treatment issue when neuroprotective agents are available: Should hemizygous individuals take such drugs? Finally, more AR loci and genes could be uncovered: One recent study eliminated *parkin*, *DJ-1*, and *PINK1* mutations in up to 40% of the participating AR PD families [14].

18.2.1 Causative Mutations

18.2.1.1 PARK1 and PARK4: SNCA. PARK1 AD PD was initially reported in a large family of Italian origin (town of Contursi, southern Italy) [15]. PD in the Contursi kindred was linked to chromosome 4q21–23 [15], with subsequent identification of the A53T causative mutation in the gene for α -synuclein (*SNCA*) [16]. Two more causative point mutations in *SNCA*, A30P [17] and E46K [18], were found in independent German and Spanish kindreds.

The family originally assigned to the PARK4 locus (Iowa kindred) turned out to have a chromosome 4 triplication containing *SNCA* [19]; therefore, PARK4 is allelic with PARK1 and not a separate locus. An independent genomic triplication in a Swedish American family [20] and chromosome 4 duplications [21, 22] have also been reported, confirming a gene dosage effect in α -synuclein-based disease.

The A53T mutation causes levodopa-responsive parkinsonism with a younger age of onset, longer duration, and lower prevalence of tremor compared to IPD [23, 24]. Neuropathology includes Lewy bodies [25]. A30P-associated disease has a typical mean age of onset (59.7 years) as well as levodopa-responsive parkinsonism based on clinical records and very few available patients; no pathology is available to date [17, 26]. Both affected A30P family members and mutation carriers have impairments on neuropsychiatric testing, implying that mild cognitive impairment could be an early feature of disease [26]. In contrast, the E46K mutation causes early-onset parkinsonism with early, prominent dementia and widespread Lewy body pathology [18]. The affected family was first reported as Lewy body dementia, an example of the overlap between PD and other clinically defined disorders. There are few *SNCA* duplications cases to confirm the range of possible phenotypes, but two unrelated individuals and members of the one reported French family share many features of typical PD

[21], although some have a postural tremor [21]. Overall mean age of onset was 48, followed by a long, slow course of levodopa-responsive parkinsonism without early dementia [21, 22]. *SNCA* triplication instead causes a more fulminate disease in both reported kindreds, with onset in the early thirties of levodopa-responsive parkinsonism, rapidly progressing to early, prominent dementia; postural hypotension is also a major feature [19, 20]. Early high levels of α -synuclein-positive Lewy bodies are observed not only in substantia nigra and other brain stem structures but also in cortex, as in Lewy body dementia. Cell loss in the same areas, as well as unusual loss in hippocampus, is seen. Glial α -synuclein deposits, usually a feature of multiple-system atrophy, have been reported in samples from the Iowa kindred [27]. These contrasts between different point mutations and different gene doses, especially the overlaps with other synucleinopathies, emphasize the need for eventual change in PD nomenclature to better reflect underlying neuropathology as well as clinical distinctions [4, 28].

Mutations and multiplications of *SNCA* are rare within IPD or clearly familial disease [29]. A53T and A30P mutations were not found in large screens of Indian [30] and Chinese [31, 32] populations. The A53T mutation may be rare in the United States outside of Greek and Italian origin populations [33]. Despite this, α -synuclein has become a focus of intense scientific research. Lewy bodies and neuritis in IPD cases contain α -synuclein [34], making this rare cause of AD PD directly relevant to sporadic disease. α -Synuclein immunostaining is now used to visualize Lewy bodies in sporadic disease neuropathology [1].

α -Synuclein is an 140-amino-acid protein of unknown function [35]. It is part of a protein family that includes β - and γ -synucleins [36]. It is highly ubiquitously expressed throughout brain, enriched in synaptic terminals [37, 38]. α -Synuclein binding to lipids and specific proteins has been demonstrated; however, the purpose of these interactions is not fully understood (see [39] for a review). Speculative functions include regulating synaptic vesicles and synaptic plasticity [36, 37, 39]. α -Synuclein interacts with lipid rafts, which could help target it to synapses [40]. A role in regulating presynaptic vesicles, particularly in dopamine terminals, is supported by dopamine neurotransmission changes observed in α -synuclein knockout mice [41]. In contrast, double-knockout mice lacking both α - and β -synuclein made by a different group had decreased levels of dopamine and of some small synaptic proteins but normal neurotransmission, synaptic plasticity, and synaptic vesicles [42], implying a different, perhaps long-term maintenance role for α -synuclein in dopaminergic terminals.

Changes in α -synuclein are thought to cause disease through a toxic gain of function. There are several lines of evidence for this assertion, including the inheritance pattern of human *SNCA* mutation disease. In addition, mice with a knockout (complete loss) of α -synuclein are viable and have normal gross brain structure and basal ganglia, although detailed studies of dopamine release or dopamine levels suggest some functional loss as above [41, 42]. One potential toxic gain of function that could link *SNCA* point mutations, *SNCA* genomic multiplication, and IPD is accumulation of harmful α -synuclein forms.

The level of α -synuclein is clearly related to disease [43]. In cases of genomic triplication or duplication disease, higher α -synuclein levels correlate with more severe disease and younger ages of onset. Families with duplications have an age of onset, long duration of disease, and range of symptoms very similar to IPD, with few

exceptions, as discussed above [21, 22]. In contrast, triplication kindred members had an average onset early in their fourth decade, rapid progression, and severe clinical and neuropathological disease, including early dementia [19, 20, 44]. α -Synuclein expression levels are approximately doubled in affected genomic triplication post-mortem samples [20, 45]. Mice overexpressing human wild-type α -synuclein in neurons show some loss of dopaminergic nerve terminals and inclusion body pathology [46], although this has not been replicated in all animal models [47]. Increases in α -synuclein, either absolute or relative increases in abnormal locations, could also be caused by point mutations. Both A53T and A30P mutant α -synuclein forms do not translocate into lysosomes, blocking their own degradation as well as that of other lysosomal substrates [48]. The A30P mutation disrupts α -synuclein binding to lipid rafts, causing accumulation of protein outside of synapses [40].

Too much α -synuclein may promote disease via abnormal aggregation. In vitro, α -synuclein monomers form protofibrils, which then progress to the fibrillar forms observed in Lewy bodies [49]. α -Synuclein is a remarkably plastic molecule, with the ability to exist as an unstructured monomer or form multiple different types of aggregates [50]. The normal functions and potential toxicity of different α -synuclein polymers or fibrils is under debate. The intermediate protofibrils may be the key toxic α -synuclein form. The A53T and A30P mutations have opposing effects on fibril formation, with A53T increasing fibrillization, but both increase protofibril formation, leading to speculation that toxins that increase protofibrils would worsen disease while drugs that specifically clear protofibrils may be protective [4, 51]. Protofibril formation is found in genomic triplication cases [45] as well as in vivo models with increased wild-type α -synuclein. Therefore both α -synuclein overexpression and α -synuclein mutation genotypes are capable of creating an increased protofibrils phenotype. How protofibrils go on to damage cells is unclear. They may form membrane-damaging pore structures, an effect enhanced in vitro by the A53T and A30P mutations [52].

Although it leaves behind the potential impact on synaptic regulation or other unknown α -synuclein functions, abnormal protein aggregation is an important pathogenic theory for IPD (Fig. 18.1). Aggregated α -synuclein is found in “synucleinopathies” other than IPD, including multiple-system atrophy and Lewy body dementia, suggesting that abnormal aggregation pathways will provide a pathophysiological way to define overlapping clinical disease entities. Aggregation of other proteins, such as tau and huntingtin, is a feature of several neurodegenerative disorders. Finally, protein aggregation also offers a way to tie together different toxin and genetic PD models. Increased oxidative stress, recognized as a feature of IPD (discussed below), can increase α -synuclein aggregation [53]. Possible pathogenic agents can be tested against protofibril formation to observe whether they fit into this pathogenic theory or represent unrelated neuronal damage pathways. Identifying a toxic intermediate in the aggregation process, such as protofibrils, suggests therapeutic agents: drugs that decrease protofibril formation, increase protofibril clearance, or both [4].

18.2.1.2 *PARK2: parkin*. The first reported causative AR PD mutations were found in the *parkin* gene [54] within the PARK2 locus [55]. To date, *parkin* mutations are the most common genetic cause of early-onset PD, defined as onset younger than 50 years [56, 57]. The initial mutations were identified in Japanese families described

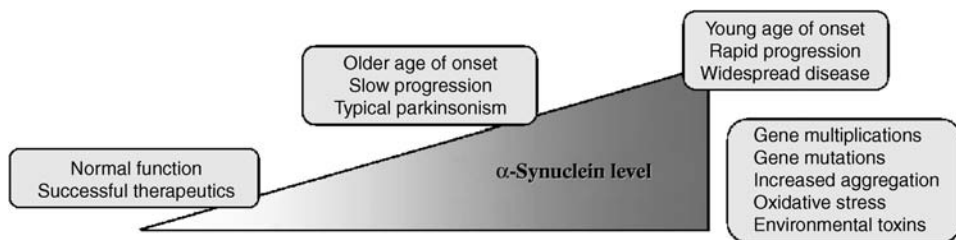


Figure 18.1 α -Synuclein toxicity is dose related. Genetic or environmental events may increase α -synuclein protein or decrease degradation to effectively increase protein, particularly protofibril, levels. Some events are clearly associated with disease: *SNCA* genomic multiplications cause increased α -synuclein expression, and *SNCA* disease-causing mutations increase protofibril formation. Genetic variability in the *SNCA* promoter, increased oxidative stress, or other environmental factors may all affect disease risk through altering α -synuclein. Lower levels of α -synuclein are associated with milder disease; for example, genomic duplication cases compared to genomic triplications. Successful therapeutics that would prevent or ameliorate disease in this scheme would decrease α -synuclein protein and protofibril levels.

as AR juvenile parkinsonism (AR-JP) [54]. Clinical features of AR-JP include mean age of onset in the late twenties, very slow progression, and levodopa-responsive parkinsonism with early dyskinesias and motor fluctuations [58, 59]. In addition, symptoms are relieved by sleep, and foot dystonia and hyperreflexia are common presenting features [58, 59]. Autopsy studies found cell loss and gliosis in substantia nigra and other structures but no Lewy bodies [59–61]. The combination of unique additional clinical features and absence of Lewy bodies led the Japanese groups to label AR-JP distinct from IPD or even inherited typical PD [58, 60], a consideration to keep in mind when reviewing subsequent attempts to link parkin protein and *parkin* mutations to general theories of PD pathophysiology.

Is *parkin* mutation disease a PD phenocopy or the same as other genetic parkinsonisms or IPD, despite cases without Lewy bodies, the neuropathological hallmark of PD? There are now two reported compound heterozygous *parkin* mutation cases with α -synuclein-positive Lewy body pathology [62, 63] and one homozygous case with atypical α -synuclein inclusions [64]. There are also cases outside the Japanese families that lack Lewy bodies; one Dutch subject had tau pathology without Lewy bodies [65]. Because Lewy bodies are central to the current definition of IPD, the scarce autopsy data on *parkin* mutation cases is cited in the ongoing debate of how *parkin*-associated disease is related to other parkinsonisms and whether disease should be classified by genotype, pathology, or other criteria.

Since the first mutation report, at least 42 different studies have identified nearly 100 different *parkin* mutations, including deletions, point mutations, and a high level of exon rearrangements (see [57] for a review). *Parkin* mutations are very frequent in early-onset AR PD, up to 50% of families in some studies [56, 57]. Early onset is more likely associated with *parkin* mutations than late; for example, mutations were found in 5% of all families screened in one large survey, 18% of the early-onset families [66]. *Parkin* mutations are also prominent in PD without known family history, especially with very early onset. One European study found homozygous

parkin mutations in 77% of isolated PD cases with age of onset 20 or younger but in only 3% of cases over 30 [56].

These mutation studies have expanded the clinical and pathological phenotypes associated with *parkin* genotypes. As the (rare) late-onset sporadic PD cases with *parkin* mutations demonstrate, clinical features can be nearly identical to typical IPD. One South Tyrolean kindred carries a compound heterozygous *parkin* mutation causing late-onset, tremor-predominant PD [63, 67]. Other groups report symmetric onset, dystonia at onset, diurnal fluctuations, and hyperreflexia. The range of phenotypes continues to widen as large screens pick up new mutations. Both genotype and age of onset can be used to help predict the overall phenotype [68], although phenotypic variability makes this an inexact process at best [63, 69]. Better understanding of genotype/phenotype correlations may help classify patients and shed light on how *parkin* protein changes contribute to pathology.

At about 1.5 Mb, *parkin* is one of the largest known genes [54]. Most mutations affect exons 2 and 7, but disease-causative mutations occur in all exons [57]. The gene size and mutation complexity, on top of the phenotypic variability, make genetic testing expensive and difficult. Despite the relative frequency of *parkin* mutations, especially in early and very early onset groups, genetic testing requires labor-intensive gene analysis and careful genetic counseling. Attempts to determine founder effects and mutational hot spots in particular populations may help simplify this process in the future [57].

A further complication is the potential role of hemizygous mutations (one mutated and one normal *parkin* allele). Although inherited PARK2 disease is so far overwhelmingly AR, there are several reports of small families and single affected individuals carrying hemizygous *parkin* mutations [57, 62, 68, 69]. A very small positron emission tomography (PET) study of members of the South Tyrolean family (phenotype unusually similar to IPD) found minor but significant decreases in dopamine uptake in all striatal regions in hemizygous family members compared to controls [70]. Hemizygous mutations may increase susceptibility for parkinsonism [62, 63], although their role in disease remains an area of active debate [71].

Parkin is an E3 ubiquitin–protein ligase [72, 73], part of the proteasome pathway [74]. Chains of ubiquitin added to misfolded or damaged proteins are recognized by the proteasome, which then degrades the targeted proteins. E3 ligases mediate the transfer of poly-ubiquitin chains from E2 enzymes to specific protein targets [74]. Parkin contains two RING (really interesting new gene) finger domains, needed to bind E2 enzymes and protein targets, and an IBR (in between RING fingers) domain [54]. In addition, there is an ubiquitin-like domain in the *parkin* N-terminus that interacts with the proteasome [75]. Mutations have been reported in all of these domains [54, 57, 75]. Loss of *parkin*'s E3 ligase function, via actual loss of ligase activity or other inability to degrade protein substrates, is thought to cause AR *parkin* mutation disease [72, 73, 76]. Parkin may also have roles in mitochondrial function [77], nonproteasomal regulation of α -synuclein [78–82], and general neuroprotection [83].

Possible loss of *parkin* function in IPD is under active investigation. A recent animal model of PD uses systemic proteasome inhibitor exposure to create progressive dopaminergic (and other) neuronal degeneration in rats, demonstrating the potential link between proteasome dysfunction and sporadic PD pathogenesis [84]. One way that wild-type *parkin* could be inactivated in sporadic disease is via nitric

oxide exposure [4]. Nitrosylation damage to parkin is seen in human PD, animal model samples, and in vitro where it can alter parkin function [81, 85]. Free radicals, including NO-derived species, mediate a toxic exposure form of parkinsonism [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)], discussed below. Therefore, damaged inactivated parkin could be created in oxidative stress-mediated PD pathophysiology, tying parkin to IPD. In addition, a possible parkin function in mitochondrial-mediated apoptosis intersects with theories of IPD pathogenesis. In vitro, parkin can prevent mitochondrial release of cytochrome *c* and subsequent apoptosis [77]. Some animal models of parkin disease (genetic knockouts lacking *parkin*) have defects in mitochondrial function [86–88]. Although genetic animal models have so far not replicated key features of human disease, particularly loss of dopaminergic neurons, these observations directly overlap with the mitochondrial dysfunction theory of PD pathogenesis, discussed below. Therefore, both loss of E3 ligase activity and potential extraproteasomal parkin functions could intersect with IPD pathophysiology.

Several parkin substrates have been identified in vitro (reviewed in [4, 39]). Characterizing parkin substrates, and their behavior when overexpressed or not degraded, is one way to trace parkin-mediated pathology. It is also a route to connecting parkin to α -synuclein pathology. For example, some putative parkin substrates are synaptic proteins [4, 39], and parkin is found in postsynaptic densities, colocalized with lipid rafts [89]. α -Synuclein, a synaptic protein, also interacts with lipid rafts [40] and has potential roles in synaptic regulation (discussed above).

Direct degradation of α -synuclein by parkin has not been demonstrated; however, parkin can protect cells from damage by mutant α -synuclein or overexpression of wild-type α -synuclein by an unknown, possibly nonproteasomal, mechanism [78–82]. In addition, parkin does directly interact with and ubiquitinate synphilin-1, an α -synuclein-interacting protein [90]. Parkin protects cells from combined α -synuclein/synphilin-1 overexpression toxicity induced by proteasome inhibition [81]. Interest in synphilin-1's role in PD has increased with a report of a point mutation in *synphilin-1* found in two apparently sporadic PD cases but not several hundred controls [91]. Detailing parkin, synphilin-1, and α -synuclein interactions may define pathogenic mechanisms and neurotherapeutic targets in both PARK1/PARK4 and PARK2 genetic disease and IPD [39].

18.2.1.3 PARK6: PINK1. The PARK6 locus was initially mapped to chromosome 1p35–36 in a large consanguineous Sicilian family with AR parkinsonism [92]. Subsequent refinement of the region using other European [93] and Asian [94] families and sequence analysis of candidate genes [95] identified two homozygous mutations in the *phosphatase and tensin (PTEN)-induced putative kinase 1 (PINK1)* gene [95].

The PTEN pathway is involved in tumor suppression; PTEN is a cytoplasmic phosphatase with many potential downstream targets. PINK1 was first described in a study aimed at identifying genes transcriptionally transactivated by *PTEN* [96]. Although PINK1 was upregulated by exogenous *PTEN* and decreased in ovarian cancer cells, the study did not show any growth-suppressive effect of PINK1 itself. Its putative kinase activity was assigned based on sequence homology to the serine/threonine protein kinase catalytic domain [96]. Both of the first reported *PINK1* mutations affect the putative protein kinase domain: G309D changes a highly

conserved amino acid position; W437OPA truncates off the last 145 amino acids of the C-terminus [95]. Disease-causing mutations in non-European families (Japanese, Taiwanese, Israeli, Filipino) also include homozygous point missense mutations and truncations in the protein kinase domain as well as compound heterozygotes [14, 97]. Reported disease-causing mutations now include missense, nonsense, insertions, and deletions [14, 95, 97–100] but only rare examples of homozygous mutations outside the protein kinase domain [98]. There is a much lower rate of exonic deletions compared to *parkin* mutations, likely due to the much smaller size of *PINK1* [14].

Large cohort screens for *PINK1* mutation types, prevalence, and related phenotypes are still underway. One large North American series identified two probands (one Filipino), both young onset with AR family inheritance patterns: one homozygote and one compound heterozygote, all mutations in the protein kinase domain, none found in the initial 180 control samples or in late-onset sporadic PD cases [99]. Several other sequence variants, not predicted from location to have a clear biological impact, were too scarce in both PD and controls to statistically show enrichment in affected or unaffected groups. Interestingly, further screening of Filipino controls for the homozygous Leu347Pro mutation found heterozygous Leu347Pro mutations in 3 of 50 subjects, implying a prevalence of 1 in 4000 for AR disease associated with this mutation in this population [99]. This finding adds to smaller family-based studies of Asians and Filipinos [14, 97], suggesting a particular *PINK1* role in AR familial disease in these specific populations.

PINK1 mutations may also have a larger role in early-onset sporadic Italian PD cases compared to Northern European and North American populations [100, 101]. Screening 100 Italian early-onset (<50 years) PD patients and 200 controls uncovered 2 patients (no controls) with homozygous or compound heterozygous *PINK1* mutations [100]. The highest *PINK1* mutation prevalence figures are from a genomic sequencing screen of consecutive sporadic early-onset PD, controls, and familial PD cases with AR inheritance previously screened for *parkin* and *DJ-1* mutations [101]. Four of 90 sporadic PD cases, all Italian, carried homozygous missense or truncating mutations. Although the number of Italian samples was much larger than any other group in this study, mutations were only found in Italian sporadic cases [101]. Unlike the Asian and Filipino sample data, the only mutations observed in familial cases were hemizygous and did not segregate with disease [101].

Another 6 Italian sporadic patients in the same study carried single heterozygous (hemizygous) changes, including two intronic mutations of unclear significance, and none observed in prior homozygous disease cases [101]. In contrast, 120 fully or partially sequenced Italian control samples yielded 4 hemizygous changes: one truncating, two 5'-untranslated region and one silent exonic change. The Valente study also found a higher incidence of hemizygous *PINK1* mutations in patients (5%) compared to unaffected controls (1%) [100]; however, the small number of patients screened makes the significance of this finding uncertain. A large prevalence study in Ireland uncovered a single hemizygous *PINK1* mutation outside the protein kinase domain in one (age of onset 51) of 290 PD patients, not found in 2224 control chromosomes [102]. In both the Irish and Italian studies mutation groups were usually located in regions of *PINK1* with unclear if any functional significance [100, 101].

The relevance of hemizygous *PINK1* mutations to disease or disease risk is speculative at best given the very small numbers of any particular heterozygous

change thus far. The data are much weaker than that for *parkin* mutations (above). In hemizygous patients, undetected intronic or promoter mutations in the other chromosome may account for disease or the patients may be *PINK1* mutation carriers with PD from another cause. In the Irish and Italian studies above, exon copy dosage was unchanged in all hemizygotes, in contrast to the genomic rearrangements and exon dose alterations seen with *parkin* and *DJ-1* [100, 102]. It is possible that hemizygous mutations in AR PD-associated genes increase risk for sporadic PD by creating subclinical neuronal dysfunction. A very small PET study in PARK6 families bolstered the case for increased susceptibility with some mutations: three asymptomatic hemizygous carriers had a 20–30% decrease in dorsal putamen dopamine uptake compared to controls [103].

In contrast, a linkage disequilibrium approach using targeted single-nucleotide polymorphism (SNP) analysis of over 500 each European PD patients and controls found that common *PINK1* genetic variation does not affect sporadic PD, young onset or in general [104]. A second genetic association study in an unrelated (Finnish) case–control series also found no role for *PINK1* genetic variation in late-onset sporadic PD [105]. Finally, a case–control association study of three common *PINK1* variants in Canadian samples found no effect on early-onset PD [106]. Taken together, these studies indicate that *PINK1* mutation does not influence nonmendelian PD forms in European and other Caucasian populations. Even with this evidence, speculation about a role for hemizygous mutations in AR PD-associated genes is likely to continue until we have a better understanding of the function of PINK1 and other protein products in disease pathogenesis, enabling biomarker development for true subclinical and early disease.

The most common *PINK1* mutation phenotype, usually associated with missense mutations, is extremely similar to sporadic PD except for an earlier age of onset. Age of onset is mostly in the third to fourth decade, with a very slow course of typical asymmetric levodopa-responsive disease, including development of dyskinesias [14, 95, 100, 107, 108]. Rare patients in the original families had dystonia at onset or psychiatric features [94, 97]. The phenotype has been extended by case reports of novel *PINK1* mutations. A homozygous insertion mutation creating frame-shift and truncation outside the protein kinase domain produced a very early onset phenotype with dystonia and sleep benefit, closer to *parkin*- and *DJ-1*-associated phenotypes [98]. A homozygous protein kinase domain deletion also causes a *parkin* mutation-like early dystonia and sleep benefit picture as well as dementia [14]. The range of motor, cognitive, and psychiatric features is likely to grow as patients and families with known *PINK1* mutations are followed longitudinally.

As with mutation to phenotype correlations, the functional consequences of individual mutations are still being defined. Most, but not all, mutations occur in the predicted kinase domain, where they could potentially interfere with substrate interactions or enzyme activity (reviewed in [101]). PINK1 does demonstrate kinase activity in vitro [96]. In addition to the putative serine/threonine protein kinase domain, the ubiquitously expressed PINK1 protein also includes a predicted mitochondrial targeting motif [100]. Both wild-type and mutated PINK1 localize to mitochondria in in vitro transfection systems, and cells transfected with mutated or wild-type *PINK1* exhibit normal mitochondrial function at baseline [100].

Compared to cells expressing wild-type PINK1, cells transfected with mutated *PINK1* were less able to sustain mitochondrial function and more likely to undergo

apoptosis in the face of a single toxin-induced mitochondrial injury [100]. Thus it is tempting to tie *PINK1* mutations into theories of mitochondrial dysfunction in IPD and mitochondrial pathways for *parkin* and *DJ-1* mutation effects. However, the toxin used, a peptide compound, also inhibits proteasome function [100], another prospective PD pathogenesis player given *parkin*'s major known function and effects of proteasome inhibitors in animal models. Untangling *PINK1* roles in these possible disease pathways is a major goal of current research.

In summary, *PARK6/PINK1* mutation disease, while rare, is worldwide, with initial studies indicating a higher frequency in Asian familial, Filipino, and Italian early-onset sporadic disease [14, 97, 99–101]. Unlike other AR PD-associated genes, *PINK1* does not demonstrate much genetic rearrangement, and recent studies build a strong case that it does not have a role in nonmendelian disease [104–106]. Mutations are most often seen in the protein kinase domain. *PINK1* could normally protect cells from mitochondrial damage or proteasome inhibition [100], roles yet to be confirmed.

18.2.1.4 *PARK7: DJ-1*. *PARK7* is close to but clearly separate from *PARK6* on chromosome 1 [109]. Two different homozygous mutations, a large deletion in a Dutch family and a missense mutation in an Italian kindred, were found in the *DJ-1* gene [110, 111]. Subsequent large screens of early-onset PD in different populations have uncovered compound heterozygous as well as homozygous disease-causing mutations, including missense, frame shifts, splice site alterations, and deletions [111–114].

DJ-1 mutations are a rare cause of early-onset AR PD, accounting for an estimated 1% of cases [112, 113, 115], low even in comparison to *PINK1*- and *parkin*-associated disease in studies to date [116, 117]. As with *parkin*, genomic rearrangements occur in *DJ-1*, requiring vigilance to detect any gene dose or promoter changes that could be the second mutations in apparently hemizygous carriers [114]. Even when these methods are employed in early-onset PD samples, *DJ-1* mutations remain rare [115]. Unlike *PARK2/parkin*, *PARK7* has not been implicated in late-onset familial or sporadic PD [114, 118]. As with *PINK1*, the significance of single, hemizygous *DJ-1* mutations is uncertain but likely low. Although population data for all reported mutations are not yet available, evidence so far does not support a role for hemizygous mutations in PD susceptibility [111, 118]. Full exploration of this question is still underway.

Affected individuals in the initial families all had an early age of onset (< 40 years) of fairly typical slowly progressive levodopa-responsive parkinsonism [109, 111]. This is very similar to *PINK1* mutation disease; *parkin* mutation disease has a more variable age of onset (above). Postural tremor, off dystonia, bletherospasm, severe anxiety, and psychotic episodes were reported in some *DJ-1* mutation cases [109]. Severe anxiety was also reported in two other patients [112] and a single Turkish patient had depression without cognitive deficits [111]. The significance of the behavioral changes in particular is unclear given the small number of cases. Autopsy data are not available. *DJ-1* is not found in Lewy bodies in sporadic PD but has been reported in α -synuclein-positive inclusions in multiple system atrophy and tau-positive inclusions [119, 120], making it difficult to predict pathological findings in *DJ-1* mutation cases. Single-photon emission computed tomography (SPECT) and PET studies on a very small number of individuals demonstrate severely decreased presynaptic dopaminergic function in homozygous *DJ-1* mutation carriers [109, 111].

The ubiquitously expressed *DJ-1* gene was first identified in screens for potential oncogenes [121, 122]. The DJ-1 protein was independently identified as a GAPDH interactor and part of a messenger RNA (mRNA) binding complex [123], providing a potential link between DJ-1 and neurodegeneration [114]. GAPDH immunoreactivity has been reported in α -synuclein-positive Lewy bodies [124], and GAPDH may play a key role in neuronal apoptosis [114]. Several other normal functions have been proposed based on sequence homologies and in vitro assays, including weak protease and chaperone activities, all yet to be confirmed [4, 39, 114].

DJ-1 exists as a cytoplasmic dimer in cell culture [111, 125, 126]. The effects of mutations on protein localization in vitro vary between studies using different cell culture systems. Some report that the L166P mutation shifts DJ-1 to mitochondria [110], versus observations of both wild-type and mutant protein in cytoplasm and mitochondria [126]. Others report a cytoplasm-to-nucleus shift for L166P and E64D mutants [111]. Normal and pathological localization of DJ-1 provides clues to possible functions: transcriptional regulation if moved to the nucleus, for example [114]. The reports of mitochondrial localization, both baseline and induced by mutations or oxidative stress in some in vitro systems [110, 126, 127], are especially intriguing given the growing importance of mitochondria in neurodegenerative disease (parkin and PINK1 discussed above, mitochondrial toxins below).

DJ-1 is one of a few proteins that become more acidic with oxidative stress [127, 128]. Oxidation of a cysteine residue has been proposed to trigger movement of DJ-1 to mitochondrial outer membranes [127] and to confer antioxidant activity via direct scavenging of free radicals [129], both still speculative outcomes. Mutations at the cysteine residue that block oxidation also render cells more vulnerable to mitochondrial damage [127]. The PD-associated mutation L166P increases oxidative stress damage in cell culture, probably through instability and loss of DJ-1 [129].

A normal protective role for DJ-1 fits with AR inheritance and likely loss of function in disease. These preliminary findings tying DJ-1 to oxidative stress and mitochondrial function converge with mechanistic pathways common to both genetic and environmental PD risks [130] (Fig. 18.2). However, the physiological function(s) of DJ-1 is far from settled. Other data connect DJ-1 to parkin, proteasomal function, and tau pathology, that is, abnormal protein aggregation [4, 114, 125]. Sorting out connections between DJ-1 and other PD-associated proteins will provide much needed clarification of DJ-1-associated disease pathophysiology and therapeutic targets.

18.2.1.5 *PARK8: LRRK2*. An AD form of parkinsonism with about 70% penetrance, late onset, and fairly close clinical similarity to IPD was initially linked to chromosome 12p11.2–q13.1 in a large Japanese family [131]. Subsequent independent confirmation of the locus in European families [132] and identification of pathogenic mutations in the novel leucine-rich repeat kinase 2 (*LRRK2*) gene in multiple families (below) indicate that *PARK8* is a major locus for AD PD, responsible for much more disease than *PARK1*.

The first *LRRK2* mutations were reported by two groups independently [133, 134]. One missense mutation segregated with *PARK8*-linked PD in Basque families; the reporting group named the associated novel protein dardarin, from the Basque word for tremor, *dardara* [133]. A total of six missense mutations and one putative splice site mutation were reported, some in a highly conserved putative kinase

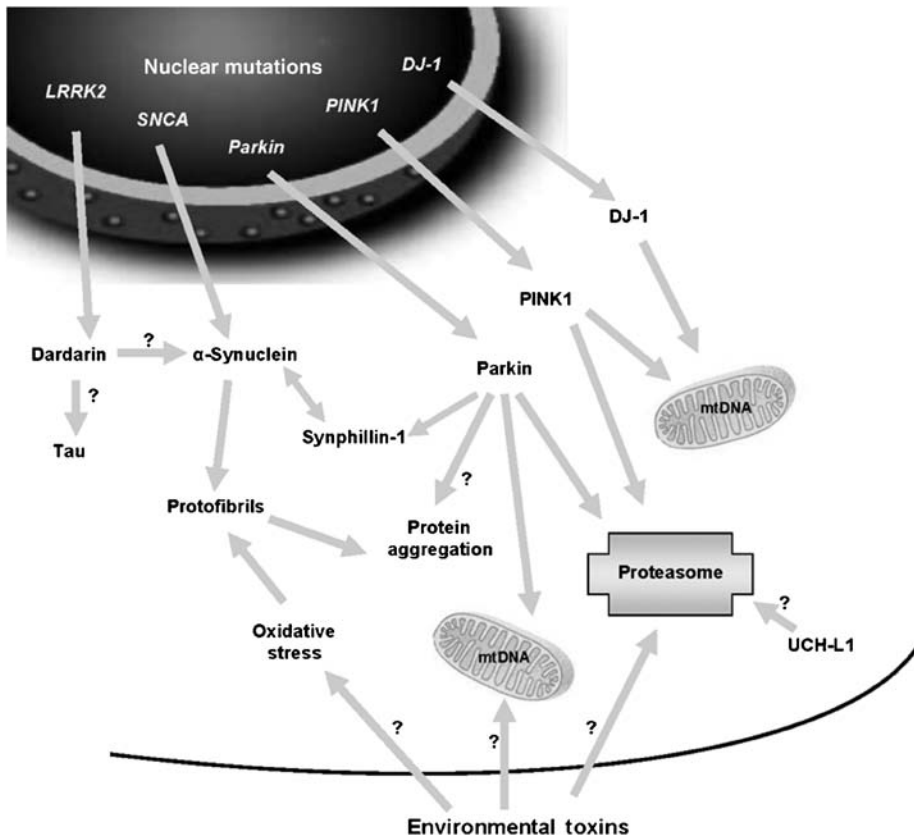


Figure 18.2 Proposed pathogenic mechanisms in PD. Mutations in a few specific genes cause monogenetic parkinsonisms. The associated protein products interact with protein aggregation, the ubiquitin-proteasome system (UPS), mitochondria, and oxidative stress in interconnected pathways. Mutations in *LRRK2* are the newest and possibly most prevalent source of monogenetic PD. Dardarin is speculated to interact with α -synuclein and other aggregating proteins, such as tau, but its function is still unclear. α -Synuclein aggregation, particularly protofibril formation, may underlie PARK1/PARK4 disease as well as idiopathic PD. Parkin, an E3 ligase in the UPS, definitely interacts with the proteasome. It may also have a role in mitochondrial function and connect to α -synuclein via interaction with synphilin-1. Mutated synphilin-1 might cause disease itself. UCH-L1, another UPS protein, has an unconfirmed role in inherited PD; mutation of *UCH-L1* is designated PARK5. PINK1 may normally protect cells from mitochondrial injury or proteasome inhibition. DJ-1 is also associated with mitochondria. It may move to mitochondria as a response to oxidative stress. Mitochondria are central to several of these pathways. Changes in mitochondrial DNA (mtDNA) could influence susceptibility to PD. Environmental exposures may contribute to pathology at several levels, including mitochondrial complex I inhibition, other increases in oxidative stress, and proteasome inhibition. (See color insert.)

domain [133, 134]. The original Japanese PARK8 family harbors one of these *LRRK2* missense mutations [135].

The *LRRK2* gene contains 51 exons [133, 134]. Dardarin/*LRRK2* is a protein of unknown function. The predicted amino acid sequence contains several highly

conserved areas, including a Roc domain [Ras/guanosine triphosphatase (GTPase) superfamily] and a COR domain (C-terminus of Roc), which potentially place dardarin in the ROCO protein family [136]. In addition, the predicted protein contains a leucine-rich repeat, a tyrosine kinase catalytic domain, and a WD40 domain [133, 134]. The kinase domain sequence may make dardarin a member of the mitogen-activated protein kinase family MAPKKK [134].

Mutations G2019S and I2020T are in or near the kinase activation segment [134, 135]; a gain of function kinase activation is speculated to account for AD disease transmission in these families. Because mutations have been reported in multiple different potential functional domains [133, 134, 137, 138], both the native and the toxic functions of dardarin remain obscure.

How and when to screen for *LRRK2* mutations have become controversial. Two clearly linked missense mutations (other mutations segregated with disease in small families) were not found in 1000 control and 300 IPD samples [134]. None of the mutations were found in 188 Japanese IPD samples [135]. In contrast, one mutation was observed in about 8% of Basque PD samples screened, half with no family history [133], making this a possible contributor to sporadic PD but only in a particular population.

Subsequent reports of relatively high *LRRK2* mutation incidence in familial parkinsonism and IPD raised the profile of dardarin in PD pathogenesis. The G2019S missense mutation was observed in multiple small European and North American families, likely from a common founder [137]. AD transmission was seen when a family history was present, but some positive samples were found in an IPD population screen. Of note, the mutation was associated with highly variable age-dependent penetrance [137]. The same mutation was identified in 4 of 61 families from a wide range of ethnic backgrounds, with a large range of age of onset and clinical phenotypes [139]. Given early reports of 2.8–6.6% of familial parkinsonism [137, 139, 140] and 1.6% of IPD [141], some groups advocated screening for G2019S in *LRRK2* as part of PD genetic testing and possible consideration of clinical use [140, 141].

Using *LRRK2* mutation screening on a clinical basis must be tempered by several factors: highly variable penetrance for different mutations, from 100% [133] down to 17% at age 50 [137]; different reports of frequency for the same mutation in referral versus community-based clinic populations [138, 142]; and the as-yet uncharted range of disease-causing mutations in terms of exon location and mutation type. This last hurdle is emphasized by identification of new mutations in screens of multiple exons [138], instead of screens restricted to exon 41 (containing the G2019S mutation) [140, 141]. Using multiple exons, the total *LRRK2* mutation rate in a community-based population was 1.6%, with G2019S representing only half of those mutations [138]. Assessment of nine *LRRK2* mutations in a community-based IPD population including 786 probands and 278 unrelated controls yielded six (0.8%) mutations [142]. Clearly, comprehensive screens of the *LRRK2* coding region (at least) in large, varied populations will be necessary before genetic testing of individual *LRRK2* mutations becomes clinically informative.

Detailed correlation of *LRRK2* mutations with clinical and pathological phenotypes is also crucial to complete. The phenotypic variability reported so far is another key factor rendering clinical use of *LRRK2* screening premature. *LRRK2* mutations are often associated with typical late-onset PD, as seen in screens of clinic-based PD

populations [18]. In AD the G2019S mutation is associated with levodopa-responsive PD with unilateral, often lower extremity, tremor at onset, slow progression, variable age of onset, and increasing penetrance with increasing age of onset [137, 143]. The Basque, European, British, and Nebraska families also present with a later, albeit variable, age of onset, mean around 65 years, and typical parkinsonism despite covering a range of genotypes [132, 144–146]. However, there are some cases with early dementia and amyotrophy in addition to parkinsonism [134]. Foot dystonia and behavioral disorders were prominent features in some members of one large British kindred [147].

The original PARK8 family lacks Lewy body pathology [131], also noted in some unrelated cases [134, 145]. Other cases have variable neuropathic findings, including mixes of tau pathology, α -synuclein-positive Lewy bodies and Lewy neurites in substantia nigra and other structures, and neural loss within single individuals [134, 145, 147, 148]. Entirely distinct pathological subsets are also seen within families even with relatively consistent clinical PD presentations [134, 145, 147, 148]. Given the range of pathological and clinical findings, some groups propose a role for dardarin in multiple neurodegenerative diseases [134], although a recent genetic screen identified the G2019S mutation in PD cases only, not multiple-system atrophy, progressive supranuclear palsy, or dementias [144]. A general role for *LRRK2* in neurodegeneration is still being debated.

Despite the potential complexity of genetic–phenotypic correlations, PARK8 is clearly an important contributor to PD risk. Work on later onset monogenetic *LRRK2* parkinsonism underscores the potential overlaps with sporadic PD; late-onset affecteds may die of other causes, obscuring family history even in AD inheritance. The range of dardarin-associated pathology also provides a tantalizing opportunity to tie together pathogenic mechanisms in a range of neurodegenerative disorders, from PD to other synucleinopathies and tauopathies. Given the highly variably pathology findings, speculation that dardarin phosphorylates proteins central to neurodegeneration, such as tau and α -synuclein, while consistent with the predicted dardarin amino acid sequence and the few neuropathology cases on record, remains entirely conjecture, as the groups reporting the original mutations are careful to point out [133, 134]. Detailing normal function and possible toxic gains of function for dardarin/*LRRK2* will be a major component of investigation into PD pathogenesis and future therapeutics.

18.2.2 Potential Causative Mutations

18.2.2.1 PARK5: *UCH-L1*. A heterozygous I93M mutation in the gene encoding ubiquitin carboxyl terminal hydrolase L1 (*UCH-L1*) was identified in two individuals in a single German family [149]. The role of the *UCH-L1* gene in PD is uncertain. Further studies have failed to find this or any other *UCH-L1* mutation in PD [150, 151] or multiple-system atrophy [152]. A meta-analysis supports a potentially protective role for a different *UCH-L1* variant, S19Y [153], although results of individual studies are so far mixed.

Discussion of *UCH-L1* remains open in part because *UCH-L1* may have normal roles in the ubiquitin–proteasome system, definitely linked to PD via *parkin* mutations. *UCH-L1* may act as an ubiquitin protein ligase [154]. *UCH-L1* is also a member of the deubiquitinating enzyme family responsible for releasing ubiquitin

monomers from polyubiquitin chains via hydrolysis [155]. Both potential UCH-L1 enzyme activities may have roles in PD pathogenesis [154]. UCH-L1 is found in IPD Lewy bodies [156]. Further functional studies and confirmation of mutations, causative or protective, in more individuals are necessary to understand the role of PARK5/*UCH-L1* in PD.

18.2.2.2 Mutations Outside of PARK Loci. The report of a *synphilin-1* point mutation in two apparently sporadic PD cases, suggesting a causative role for *synphilin-1* mutation in PD [91], is discussed above in the PARK2 section. Synphilin-1 may represent a functional bridge between parkin and α -synuclein interactions [39]. The single study to date is solid; however, *synphilin-1* mutation contributions to disease must still be confirmed.

A single study reported two changes in exon 1 of the *Nurr1* (*NR4A2*) gene, encoding a transcription factor important in dopaminergic neuron genesis and development [157], in 10% of PD samples, one-third of German ancestry [158]. Neither these nor any other sequence changes were detected in follow-up screens of exon 1 in 44 familial PD cases (United Kingdom) [159] and microsatellite marker screening of 12 families [160]. In addition, these changes were not found in a screen of 424 PD patients, 55 with a positive family history, and over 80% of German ancestry [161]. The same study found similar rates in PD and controls of an intronic polymorphism [161], conflicting with previous reports of some association of this polymorphism with disease [162, 163]. Although *Nurr1* function is critical to dopaminergic neurons, the failure to replicate findings or identify linkage in a single large family makes *Nurr1* mutation association with PD questionable at best.

18.2.3 Parkinsonism Loci

Three PARK loci without identified mutations are currently considered PD related (Table 18.1). They include a traditionally defined locus associated with distinct families as well as loci identified with newer large population analysis methods. A fourth locus, PARK9, is no longer considered PD related, although the exact associated genotype and pathology are not known, leaving the door open for mechanistic connections to other syndromes in the future. It is included here as an example of how an inherited disease can move away from its initial classification.

PARK3 is linked to families with an inherited parkinsonism syndrome; as with most mutation-associated diseases above, these families display a wider range of clinical and neuropathological features than classic IPD. The relationship of this rare disorder to IPD or other neurodegenerative diseases awaits identification of causative genetic mutations and associated proteins and continued characterization of clinical and neuropathological features. Classification of genetic diseases as PD, especially with little pathological data or specific mutations, remains controversial. In the future, PARK3 may move away from the other PARK loci, as with PARK9, or tie in closely via similar pathogenic mechanisms.

Two newer loci, PARK10 and PARK11, were identified using large population screening approaches to uncover potential genetic risks for PD. Both loci were found using autosomal dominant models and so are listed under potential monogenic causes for now.

18.2.3.1 *PARK3*. The autosomal dominant *PARK3* locus was linked to chromosome 2p13 in two American families of northern European descent, possibly with a common founder [164, 165]. The initial report noted a later (mean 61 years) age of onset and features close to IPD [164]. Long-term follow-up of the families uncovered dementia and postural tremor in affected people in addition to typical PD features [165, 166]. A single autopsy case revealed Lewy bodies in brain stem nuclei, including substantia nigra, as well as amygdala and forebrain, not seen in a single unaffected family member brain [148]. In addition, neurofibrillary tangles and plaques have been reported, implying a spread of pathology outside the typical PD range [165]. The probable low penetrance of *PARK3* (less than 40%) [164] and later age of onset are similarities shared with the *PARK8* locus; however, the range of clinical and neuropathological features is still being defined, and the incidence of the locus in European familial disease is so far very low.

18.2.3.2 *PARK9*. Kufor Rakeb disease (KRD), initially described in an Arab family, includes pyramidal signs in addition to levodopa-responsive parkinsonism [167]. Magnetic resonance imaging (MRI) showed atrophy of the globus pallidi as well as pyramids [167]. Linkage analysis of KRD identified a locus on 1p36, in the previously assigned *PARK9* location [168]; a causative mutation has not yet been identified. The disease is autosomal recessive, with juvenile onset and a subacute course. Although KRD includes parkinsonism with an initial levodopa response and recently described peak-dose dyskinesias and wearing off in the long term [169], the associated signs and symptoms are outside the realm of classic IPD. These include dementia, supranuclear gaze palsy, mini-myoclonus, visual hallucinations, and oculogyric dystonic spasms [167, 169]. Early reports noted similarities to the clinical syndrome of “pallido-pyramidal disease” (PPD), but parkinsonian and other symptoms place KRD out of that category as well. It currently remains a rare entity associated with the *PARK9* locus.

KRD provides an example of how careful long-term clinical and neuropathology follow-up can clarify the categorization of a syndrome featuring levodopa-responsive parkinsonism, even within the current evolving framework of clinical definitions of disease. The *PARK9* location, in a mutational hot spot on chromosome 1, near *PARK6* and *PARK7*, is intriguing. While *PARK9* classification awaits exact genotype and pathophysiological information, for now it is outside the PD-associated list.

18.2.3.3 *PARK10*. *PARK10* was defined by allele-sharing methods using a large Icelandic genetic and clinical database [170]. This chromosome 1p32 region is described as a susceptibility locus for late-onset PD [170]. There is no current linkage to a single family or refinement of the locus to a specific gene or small candidate gene set.

18.2.3.4 *PARK11*. *PARK11* on chromosome 2q36–37 is another locus defined in a large population using AD models [171]. Although *PARK11* and *PARK10* were both tagged in a whole-genome association study [172], *PARK11* linkage was not found in a large European population [173]. Understanding the full significance of both *PARK10* and *PARK11* loci awaits further molecular genetic and neurobiological work.

18.3 GENETICS AND IPD

Active hypotheses on the neuropathology of IPD encompass both genetic and environmental factors. The use of monogenetic forms of parkinsonism to generate hypotheses about IPD is discussed above. α -Synuclein remains the classic example of a rare monogenetic disease directly linked to sporadic PD pathology; dardarin mutations may account for a significant level of what is currently labeled IPD. Further complex genetic risks for IPD are being actively sought.

An important distinction between these hypotheses is disease risk (yes/no will manifest disease; drive primary pathogenesis) versus disease modifiers (change characteristics of disease). Disease modifier work is much less established than disease risk. Disease modifiers may alter key characteristics of PD, such as the presence or severity of particular symptoms (tremor, sleep disruption), or efficacy of medications. Predicting and preventing severe nonmotor symptoms such as dementia is an increasing clinical concern. Disease modifiers may overlap with disease risk; for example, increased genetic susceptibility to a neurotoxin will increase PD risk if the person is exposed to the toxin and may also increase symptom severity in the face of continuing exposures. Currently, the main focus of disease modifier work is in age of onset.

18.3.1 Disease Modifiers: Age of Onset

Changing age of onset represents an overlap between disease manifestation and modification, as very late ages of onset could decrease manifestation of disease during an average life span. However, treating age of onset as a disease modifier, several studies implicate genetic polymorphisms in age-of-onset variation.

Some age-of-onset modifiers are outside of the known PARK loci, although they may converge with PARK loci genes as each locus is narrowed down. Genome screens (in families without known PARK loci mutations) for genes influencing age of onset have found evidence for linkage to chromosomes 1q [174, 175], 8q [174], and 2p near the PARK3 locus [174]. The chromosome 1q genes represent possible candidates for PARK10 [175]. It is currently unknown if the age-of-onset modifier gene(s) at the chromosome 2p locus will be the same as PARK3 gene(s) with disease causative mutations [174].

18.3.2 Disease Risk

Genes already involved in monogenetic PD may also play a role in sporadic PD risk. Some *SNCA* and *parkin* polymorphisms, particularly in promoter regions, may be associated with increased IPD risk, although early analyses remain contradictory [176–179]. Further work is needed to delineate the full extent of true risk-enhancing polymorphism types, locations within *SNCA* and *parkin*, and prevalence in IPD.

There are numerous attempts to quantify possible genetic risks for IPD outside the PARK loci. Most are based on a current theory of PD pathogenesis. For example, pesticide exposure may contribute an environmental risk for PD (discussed below). Some studies find increased PD risk in populations with genetic polymorphisms that would render them poor pesticide metabolizers, implying that pesticide compounds would have an increased impact in these individuals [180, 181]. Known genetic risks

could be used to design new neuroprotective agents or tailor therapeutic regimens based on individual risk of triggering particular pathogenic mechanisms. Two recent examples, *GBA* and mitochondrial DNA (mtDNA) polymorphisms, illustrate challenges to identifying complex genetic risks in PD.

18.3.2.1 *GBA* Polymorphisms. Reports of atypical parkinsonism in type 1 Gaucher's disease led a group to examine glucocerebrosidase gene mutations in Ashkenazi Jews with PD compared to healthy subjects of the same ethnic group in the same geographic region and compared to patients with AD [182]. They found a 5-fold increase in N370S mutations and a 21-fold increase in 84GG mutations in PD patients compared to controls. In addition, three PD patients were homozygous for a mild, low-penetrance mutation. A separate screening for the N370S allele only in 160 PD patients and 92 controls of Jewish background found a nonsignificant trend of 10.7% PD samples with the N370S genotype compared to 4.3% controls [183]. However, given the high prevalence of *GBA* mutations in the Ashkenazi population (1 in 17 for the most common mutation), most *GBA* mutation carriers will not develop PD. Screening *GBA* in PD cases in a different population found 5 of 88 heterozygous mutations in PD and 1 of 122 in controls, a marginal effect [184]. Functions of *GBA* do theoretically overlap with possible PARK loci disease pathways, given α -synuclein and parkin interactions with lipids, for example [185]. A genomewide screen in yeast for α -synuclein toxicity enhancers yielded a large number of genes associated with synaptic vesicle transport or lipid metabolism [186]. Overall the possible pathogenic link between *GBA* mutations and PD remains unclear. This interesting correlation may provide future insight into disease-modifying genes in PD and open up a novel area (lipids) for therapeutics.

18.3.2.2 Mitochondrial DNA. Studies of mtDNA in PD are driven by potential roles for complex I inhibition and oxidative stress in IPD pathogenesis, discussed below. Work on mtDNA polymorphisms could help define connections between parkin, DJ-1, PINK1, mitochondrial toxins, and IPD. Cytoplasmic hybrid (cybrid) cell experiments suggest that defects in mtDNA contribute to IPD. Cybrid cells are created by fusing platelets, which contain mtDNA but do not have nuclei or nuclear DNA, from one source with cells depleted of all mtDNA from another source. Cybrids made with PD patient platelets have decreased mitochondrial complex I activity compared to cybrids made with control platelets, suggesting a defect in the PD patients' mtDNA [187, 188]. However, PD-causative or clearly risk-associated mtDNA mutations have not been identified. On the other hand, one study of decreased, rather than increased, PD risk identified mtDNA haplogroups, and one SNP in a complex I component, associated with a reduced PD risk compared to other haplogroups [189].

18.4 ENVIRONMENT AND IPD

Twin studies and risk analyses of relatives of IPD patients indicate a large contribution of environment in IPD pathogenesis [7–11]. Complex interaction of environmental exposures and genetic risks is now the standard explanation for PD and other neurodegenerative disorders; however, the identities and mechanisms of action of the purported environmental contributors remain unknown. Potential

environmental risks for IPD have been identified through observation of toxic exposures and neuroepidemiology. Broad risk factors such as occupation and level of education are also under study. This section focuses on some of the PD risk factors that are generating active basic science work on potential pathological and neuro-protective mechanisms.

18.4.1 Lifestyle: Smoking, Caffeine, and Alcohol

Many studies have demonstrated an inverse association between cigarette smoking and PD [190]. A meta-analysis of published studies found that PD patients were half as likely to have ever smoked cigarettes compared to unaffected individuals [191]. Conversely, the risk of PD was 60% less for current smokers compared to nonsmokers [191]. This effect is not due to early death leaving fewer smokers alive to develop a late-onset disease. This very strong inverse association holds up in family based case-control studies comparing PD patients to unaffected siblings who are more likely to share environments and more genetic information than random controls [192]. In twin pairs, risk of PD is inversely correlated with the pack-years dose of cigarette smoking, more so in monozygotic than dizygotic twins, suggesting a biological anti-PD role for cigarette smoking [193].

One hypothesis is that PD patients have a preclinical personality type that biases them to not start smoking or to quit smoking [191]. This speculation is intriguing because it implies that PD pathogenesis may start decades earlier than the motor symptom onset, but it is very difficult to prove or disprove. Another is that nicotine, or some other compound in cigarette smoke, is neuroprotective, a scientifically plausible hypothesis that is as-yet unproven [190, 193]. Finally, smoking may have secondary positive effects, such as decreasing monoamine oxidase activity [194].

Coffee drinkers also have a lowered risk of PD; again, the mechanism remains unclear. Possible regulation of adenosine A2a receptors is scientifically plausible but speculative [195]. This is a somewhat less robust finding compared to cigarette smoking, as it has been confirmed in several but not all populations [196–198]; still, it holds up in a recent meta-analysis [191]. PD risk reduction is probably an effect of caffeine in general, as other caffeine-containing substances are also associated with lowered PD risk, while decaffeinated coffee is not [195, 197, 199]. The relative risk of PD decreases with increasing coffee dose [191, 195, 199], although one large prospective study observed a U-shaped curve of coffee consumption versus PD risk in women. The loss of caffeine benefit against PD risk could be due to estrogen replacement therapy use [200]. Clarifying the gender or estrogen effects on relative PD risk remains for further studies.

Alcohol consumption has a dose-dependent effect on PD risk in some studies, with low alcohol doses conferring reduced risk and moderate or high consumption associated with the same PD risk as abstention [201]. Evidence for this effect remains mixed [196–198]. Confirmation of dosing and population effects as well as pathophysiological mechanism remains under active investigation.

18.4.2 Vitamins

The effect of vitamin intake remains controversial. Thus far individual supplements have failed as neuroprotective agents in randomized, double-blind clinical trials

[202, 203]. Dietary intake may have a larger impact than isolated supplements. A prospective observational study of two large cohorts (76,890 women and 47,331 men) found no change in risk for PD with use of tablet form vitamin E, vitamin C, or multivitamin supplements; however, there was a reduced risk for PD with consumption of foods high in vitamin E [204]. A recent meta-analysis also found a reduced risk of PD with moderate or high dietary vitamin E intake but not vitamin C or beta carotene [205].

18.4.3 Pesticides

Living in a rural environment was associated with increased PD risk in many but not all studies [198, 206, 207]. Theories of potential rural exposures, such as wellwater use, have so far shown mixed results at best in follow-up studies [198, 207]. Pesticide exposure, potentially increased in rural environments, has come under intense scrutiny due to toxic exposure theories of PD pathogenesis, discussed below.

Numerous studies have shown a positive association between pesticide exposure and increased PD risk (see [198] for a review). Some studies have refuted this finding. A potential source of this conflict is that studies may broadly define pesticides, while the actual risk-inducing pesticides are limited to ones with particular mechanisms of action. Ongoing epidemiological work aims to assess specific pesticide exposures against PD risk. One widely used pesticide class is the mitochondrial complex I inhibitors. These agents could induce neurologically damaging oxidative stress, as speculated below. Linking increased PD risk to a specific pesticide class or agent, particularly one with a biologically plausible mechanism of neurotoxicity, has not been done.

18.5 MITOCHONDRIA AND OXIDATIVE STRESS: ONE WORKING THEORY OF PD PATHOGENESIS

Theories of PD pathogenesis related to protein aggregation and proteasome dysfunction are discussed above. Another major theory of PD pathogenesis, mitochondrial dysfunction and oxidative stress, was first derived from observations of toxin-induced parkinsonism. Current neuroprotective treatment work includes antioxidant therapies in phase III clinical trials. Intriguingly, proteins associated with some monogenetic PD forms, such as parkin and DJ-1, are related to mitochondria. Oxidative stress can worsen α -synuclein aggregation. Cells expressing mutant α -synuclein have an increased sensitivity to cell death caused by oxidative stress [208, 209], and wild-type α -synuclein that has been damaged by free radicals may be more prone to aggregation [53]. Mitochondrial dysfunction and oxidative stress may therefore be a bridge between genetic and environmental risks for PD.

Other active avenues of basic research include calcium handling, mechanisms of apoptosis (programmed cell death), and glutamate neurotransmission. These are also reflected in current treatment attempts, for example NMDA (glutamate) receptor antagonists as possible neuroprotective agents in PD and other neurodegenerative diseases. None of these theories, including mitochondrial dysfunction, has been clearly adopted as causal in IPD. The mitochondrial dysfunction story is used here as an example of how ongoing work attempts to define potential mechanisms for PD.

The intent is to provide the rationale for current new therapeutics under trial, not to outline a proven mechanism of disease.

The idea of mitochondrial dysfunction in PD gained credence after an outbreak of parkinsonism caused by a synthetic opiate contaminant, MPTP [210, 211]. MPTP induces acute, permanent parkinsonism after its metabolite, 1-methyl-4-phenylpyridinium (MPP^+), is actively, specifically taken up into dopaminergic neurons [212, 213], where it inhibits mitochondrial complex I [214, 215]. Given the striking clinical and pathological similarities between MPTP-induced parkinsonism and idiopathic PD, several groups investigated complex I defects in PD. Complex I activity is decreased in PD brain [216–218]. Complex I is also decreased in platelets, muscle, and fibroblasts in PD patients, suggesting a systemic mitochondrial defect underlying this specific neurodegeneration [219, 220].

Complex I is the initial large, multiprotein complex in the mitochondrial electron transport chain (ETC). This series of four complexes generates the proton gradient used by complex V to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP) in the last step of oxidative phosphorylation, the final pathway of energy-producing metabolism in aerobic cells [221–224]. The ETC complexes move electrons down an energy gradient through tightly constrained sequential transfers between molecules. In normal oxidative phosphorylation, some reduced (electron-carrying) molecules “leak” out into the mitochondrion, contributing to the normal oxidation–reduction state of the cell.

MPTP selectively damages dopaminergic cells because MPP^+ is actively and specifically taken up into cells by the dopamine transporter [212]. Neurons that lack the dopamine transporter are unaffected. If a general dysfunction of complex I is behind PD pathogenesis, then a systemic, nonselective complex I inhibitor should also cause parkinsonism, even without MPP^+ 's selective uptake mechanism. Rotenone, an organic pesticide, is highly lipophilic, so it easily crosses the blood–brain barrier and all cell membranes. It binds to complex I in all neuronal mitochondria, without using an active selective uptake system [225]. Chronic systemic infusion of rotenone in rats [225–227] and primates [228] causes nigrostriatal degeneration. There is damage to other neuronal systems as well [229] and variable motor findings [230]. The model demonstrates that a widespread mild complex I defect can have a greater pathological impact on specific neurons.

Rotenone creates dopaminergic cell damage by increasing oxidative stress. In cell culture, whole slice culture, and an in vivo rodent model, rotenone causes increases in protein carbonyls, a marker for oxidative damage to proteins [231, 232]. A vitamin E compound, α -tocopherol, rescues levels of protein carbonyls and dopaminergic cell damage from rotenone [231, 232]. Complex I inhibition may increase oxidative stress by increasing reactive oxygen species (ROS). ROS are a subset of free radicals, a term for all molecules with an unpaired electron in an outer orbital [233]. ROS are molecules that contain oxygen with an unpaired electron, making them highly chemically reactive: ROS will aggressively nonezymatically donate electrons to other compounds or take up protons from other molecules in order to form an electron pair. Formation of an electron pair between an ROS and another compound can create a chemical bond, altering and potentially damaging the compound [233]. Mitochondria, with high normal levels of molecular oxygen and baseline “leaks” of reduced compounds from the ETC, are the major source of ROS in most cells. If electron transfer is “held up” at complex I, the normal leak of reduced molecules

increases, leading to increased ROS as these molecules interact with highly reactive molecular oxygen. In in vitro isolated nerve terminal preparations, complex I defects lead to ROS formation even at low levels of complex I dysfunction [234]. Thus, the low levels of complex I defect seen in PD and model systems may be physiologically significant. Increased lipid peroxidation [235, 236] and oxidative damage of DNA [237, 238] and proteins [239] have all been observed in PD substantia nigra samples.

Overall, extensive evidence suggests the involvement of oxidative stress in PD pathophysiology, although the mechanisms by which complex I defects and oxidative stress lead to specific PD pathology remain unclear. Increased oxidative stress from exogenous toxins could interact with α -synuclein to increase that protein's toxicity, as discussed above. Dopaminergic cells may be particularly vulnerable to oxidative stress because dopamine catabolism itself creates ROS [240–242]. The combination of high baseline ROS plus a secondary, in itself nontoxic, ROS increase might explain damage to dopaminergic neurons. Despite this, neither levodopa/dopamine toxicity nor protection of neurons (in model systems) via dopamine depletion has been conclusively and consistently demonstrated. Mitochondrial dysfunction and oxidative stress remain an active theory as PD pathogenesis work moves forward. This includes generation of potential therapeutics. For example, a recent small pilot study of coenzyme Q10 suggested there was a slower change in the United Parkinson Disease Rating Scale score in the highest dose (1200 mg a day) treatment group compared to placebo [243]. This compound is now in phase III clinical trials, necessary to confirm and detail any effect of coenzyme Q10 in PD before it can be recommended as a treatment. Coenzyme Q10 may act as an antioxidant, protecting against increased ROS, and could shuttle electrons past a complex I block to complex III, one of its normal functions in the cell. Its actual efficacy as a PD neuroprotective therapeutic is as yet unproven.

18.6 CONCLUSION

Work on PD pathogenesis has exploded in recent years, with advances in molecular genetics, neuroepidemiology, and toxin models of disease. Proteins implicated in PD through monogenetic forms of parkinsonism may interact with each other and environmental exposures to create a complex, chronic neurodegenerative disorder (Fig. 18.2). New therapeutics will exploit information on protein dysfunction, including abnormal aggregation, proteasome dysfunction, mitochondrial inhibition, and oxidative stress as well as interactions between these cellular systems and environmental PD risk factors.

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INVERTEBRATES AS POWERFUL GENETIC MODELS FOR HUMAN NEURODEGENERATIVE DISEASES

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19.1	Introduction	567
19.2	Invertebrate Model System: Salient Features	568
19.3	<i>C. elegans</i> as Genetic Model System: General Features	568
19.4	<i>D. melanogaster</i> as Genetic Model System: General Features	569
19.5	Modeling Parkinson's Disease: Dopamine Neuron Cell Death	569
19.6	Modeling Trinucleotide Repeats or Huntington's Disease	577
19.7	Modeling Alzheimer's Disease	579
19.8	Perspectives and Future Directions	582
	References	583

19.1 INTRODUCTION

In the year 2002 the Nobel Prize in Physiology and Medicine was awarded to Sydney Brenner, Robert Horvitz, and John Sulston for their establishment of the nematode *Caenorhabditis elegans* as a novel experimental model organism to explore the molecular bases of organ development and cell death. Their discoveries identified key genes involved in cell division, differentiation, and function as well as those involved in classical apoptosis and determined that these genes are highly conserved both structurally and functionally with higher organisms, including humans. Their studies provide the framework in which the complexities of vertebrate macrostructure and the nervous system can confidently be explored on a molecular level in much simpler organisms such as multicellular invertebrates, with the important benefit that the results are often directly applicable to mammalian neurophysiology [1–3]. We are now entering an unparalleled age in using genetically amendable model organisms to study the basic mechanisms of neurodegenerative diseases and to use these systems to

search for pharmaceuticals and drug targets that have direct homologs in humans. In the present review, we will discuss how the nematode *C. elegans* and fruitfly *Drosophila melanogaster* have allowed us to efficiently and effectively model a number of neurodegenerative diseases and how the opportunities presented by these organisms will increase our understanding of the molecular basis of these disorders. We also describe how this system can be utilized to identify new drug targets that can protect against neuronal death.

19.2 INVERTEBRATE MODEL SYSTEM: SALIENT FEATURES

In order for model organisms to be useful for the identification of the molecular components of human disease, the key regulatory genes and molecular pathways in the models must be conserved with mammals. The model systems should also provide significant experimental advantages over their mammalian counterparts, including simplicity of growth and manipulation, scale, and genetics [3]. Furthermore, the system should also ideally provide a fully sequenced genome that can be taken advantage of for comparative and evolutionary genomics with vertebrates. Model organisms should provide tools to incorporate forward genetics or reverse genetics. Genetic screens incorporating forward genetics allow for the identification of novel molecules or pathways involved in a particular cellular process [4]. This can be one of the most powerful attributes of invertebrate models. Forward-genetic screens using chemical mutagens are one of the most common ways to generate mutants to elucidate gene function. Reverse genetics allows one to identify quickly behaviors or pathways on which a particular gene acts. Genetic knockdown mutants, using, RNA interference (RNAi) technology in which a gene product is dramatically reduced by introducing double-stranded RNA (dsRNA) into the organism, can also provide invaluable information on the role a gene plays in a biological process [5]. Finally, all invertebrate model organisms should also provide the utility to quickly generate transgenic animals through DNA transformation.

19.3 *C. ELEGANS* AS GENETIC MODEL SYSTEM: GENERAL FEATURES

Caenorhabditis elegans is a powerful model system for genetic analysis and for exploring the molecular mechanisms of neuron development, function, and disease [6–8]. The worm genome has been sequenced and contains approximately 20,000 genes (compared with 25,000 in the human genome) [9, 10]. Its small size (1 mm long), large brood size (approximately 300 progeny from a single hermaphrodite), quick generation time (three days), and ease of maintenance in the laboratory (tens of thousands can be grown on a 90-mm agar plate coated with bacteria or grown in liquid in 96- or 384-well tissue culture plates) allow for rapid growth and an amendable system to use for experimental analysis [11, 12]. The animal is transparent, anatomically simple, and well characterized (just over 1000 cells with 302 neurons in the adult), and the ease of the generation of reporter constructs and transgenic animals allows for the quick (within three days) and facile examination of neuron protein expression, localization, or morphological changes in the living animal [13–18]. Knockout and genetic mutants can be identified within as little as a

week [9, 19]. Thousands of known mutants have also been identified and are readily available at a federally funded institution free of charge (for academia) or at a nominal cost (industry) [20]. Furthermore, since the animals can easily be mated with each other, generation of strains with several genetic mutations can easily be generated. Gene knockdown by RNAi can be obtained in most cell types in vivo or in vitro by injecting the dsRNA for the gene, by soaking the animals in dsRNA, or quite elegantly by feeding the animals bacteria that express the dsRNA [5, 21, 22]. Primary *C. elegans* cultures have also recently been optimized to allow for stable growth of embryonic cells [23]. Finally, the electrical properties of a number of cell types can be evaluated using patch clamping and other electrophysiological methods both in vivo and in vitro [18, 23].

The worm's nervous system contains almost all of the known signaling and neurotransmitter systems found in mammals [24]. Enzymes and molecular pathways involved in the production of acetylcholine, glutamate, γ -aminobutyric acid, serotonin and dopamine (DA), and a number of neuropeptides as well as the transporters involved in release and reuptake are all highly conserved [25]. Consistent with this high conservation, the worm is a valid model for neuroprotective drug discovery [26]. Most of the ligand-gated and voltage-gated ion channels are also present and functional in the worm as well as all the necessary components involved in mammalian synaptic neurotransmission [24, 26, 26a]. This remarkable similarity between the vertebrate and worm nervous systems suggests that the paradigms discovered using *C. elegans* will have significant relevance to mammalian physiology and disease.

19.4 D. MELANOGASTER AS GENETIC MODEL SYSTEM: GENERAL FEATURES

The fruit fly *D. melanogaster* is a powerful genetic tool to study neurodegenerative diseases. It is one of the oldest eukaryotic genetic models and has been utilized for almost a century to examine a variety of basic biological mechanisms, including genetic inheritance, and behavioral and developmental processes. Their small size (approximately 2.5 mm) and ease of growth in the laboratory at room temperature allow for facile experimental accessibility. One of the most attractive aspects of *Drosophila* for use as a model organism is its short reproductive cycle and large number of progeny. Females can lay over 400 eggs within 10 days and their 2-week generation time allows for quick analysis of mutant animals. The fly has only four pairs of chromosomes (three autosomal and one sex), transgenic animal production has been well developed (almost 20 years old), and the genome has been completely sequenced. The short generation time, combined with a large number of progeny makes it relatively easy to score thousands of mutants in a genetic screen in a matter of weeks.

19.5 MODELING PARKINSON'S DISEASE: DOPAMINE NEURON CELL DEATH

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease affecting more than 1% of individuals over 55 years old. The disorder results from the loss of greater than 80% of the DA neurons within the substantia nigra pars

compacta (SNpc) and the formation of protein aggregates called Lewy bodies (LBs) in a significant number of the surviving neurons [27]. The degradation of the DA neurons confers the classic triad of PD symptoms, including tremors, rigidity, and bradykinesia. Although the molecular basis of this disorder has not been identified, etiological and pathological data suggest that there is both a genetic and environmental component that causes oxidative damage and mitochondrial dysfunction [28, 29].

Many vertebrate models for PD rely on exposing the SNpc to neurotoxins that cause DA neurodegeneration. Several weeks following exposure, the animals display a parkinsonian-like syndrome. The most common neurotoxins used are 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium ion [MPP^+ (the active metabolite of MPTP)], or the insecticide rotenone. 6-OHDA and MPP^+ target the DA neurons because of their high affinity for the dopamine transporter (DAT) [29–31]. DAT, the target of a number of drugs of abuse, including cocaine and amphetamine, transports the toxin into the cell where it confers the neurodegeneration [32–34]. 6-OHDA lesioning of the DA neurons is a particularly intriguing model for PD because 6-OHDA may be an endogenous neurotoxic metabolite of DA causing or contributing to the disease [35, 36].

A number of *C. elegans* and *D. melanogaster* models have been developed that recapitulate many aspects of PD. We generated a *C. elegans* transgenic line that expresses green fluorescent protein (GFP) (behind the DAT promoter) in all eight dopamine neurons within the hermaphrodite (Fig 19.1) [6, 37]. This expression allows for the DA neuron cell bodies and processes to be easily seen under a normal fluorescent dissecting scope in vivo. When we briefly exposed (0.5–1 h) the animals to 6-OHDA, we found a time- and concentration-dependent loss of DA neuron GFP fluorescence and loss of DA neuron integrity (Fig. 19.2). Consistent with vertebrate PD models, this effect can be completely blocked by coincubation with DAT agonist (e.g., amphetamine) or antagonist (e.g., cocaine), and DAT is required for the effect [37] (Fig. 19.3, data not shown). Furthermore, the effect appears specific for the DA neurons, since the serotonergic, cholinergic, or chemosensory neurons do not appear to be affected by the neurotoxin [37]. These results recapitulate several aspects of vertebrate PD models and suggest that our *C. elegans* model could be useful in identifying proteins and pathways involved in the disease.

Genetic and epidemiological studies also indicate that the etiology of PD likely involves specific molecular pathways involved in protein aggregation and degradation. To date, seven genes have been independently identified that are associated with rare, familial forms of PD: α -synuclein, parkin, DJ-1, UCH-L1, NURR1, PINK-1, and LRRK2 [38–44]. The first gene identified, α -synuclein, is a presynaptic protein that appears to interact with synaptic vesicles and could be involved in the regulation of both dopamine biosynthesis and dopamine transporter function, although its function is largely unknown. α -Synuclein is also a major component of LBs. PD-associated α -synuclein coding mutations A30P and A53T alter the structure of α -synuclein and affect DA neuron viability in vitro and in vivo [45, 46]. Very recently a new mutation in α -synuclein K46E has been identified. It appears as though all of the mutations may interfere with the proteosomal degradation pathway [47]. In collaboration with Garry Wong at Kuopio University in Kuopio, Finland, we also generated transgenic animals expressing either human wild-type (WT) or mutant

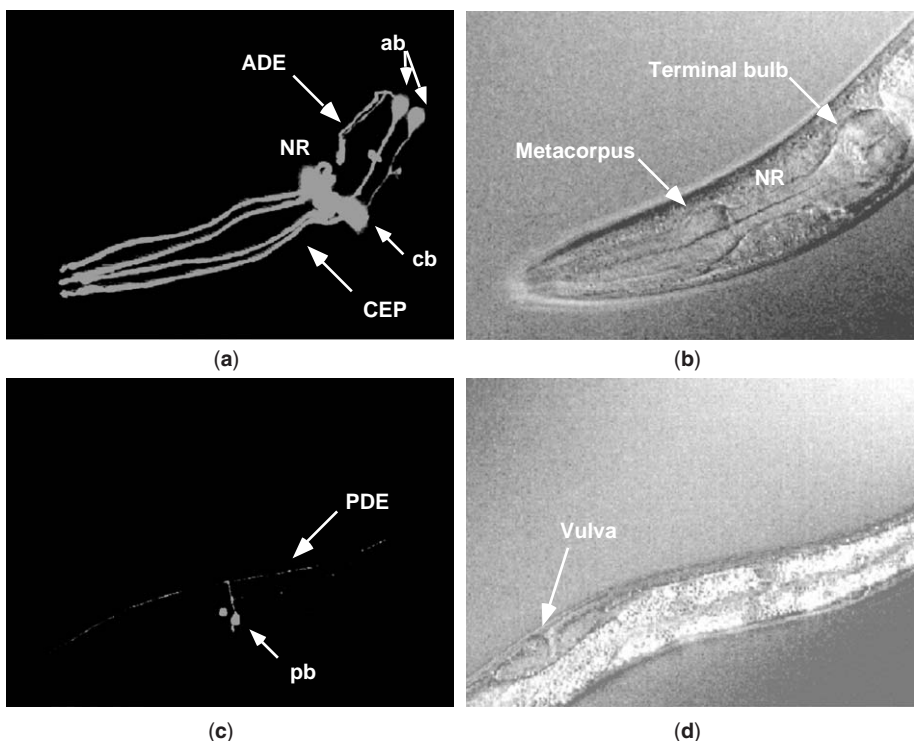


Figure 19.1 Visualization of all eight DA neurons in living, adult *C. elegans* hermaphrodites using DAT::GFP transcription fusions. (a) Three-dimensional (3D) reconstruction of confocal epifluorescence from head DA neurons in a $P_{\text{dat}:1}::\text{GFP}$ transgenic line. Arrows identify cephalic cells (CEP) and anterior deirids (ADE) processes. NR refers to the nerve ring. (b) Differential interference contrast (DIC) image of animal in (a). (c) 3D reconstruction of confocal epifluorescence of PDE neurons. Both posterior deirids (PDE) cell bodies are apparent. (d) DIC image of animal in panel (c). Anterior is to the left. (Reproduced with permission from [37].) (See color insert)

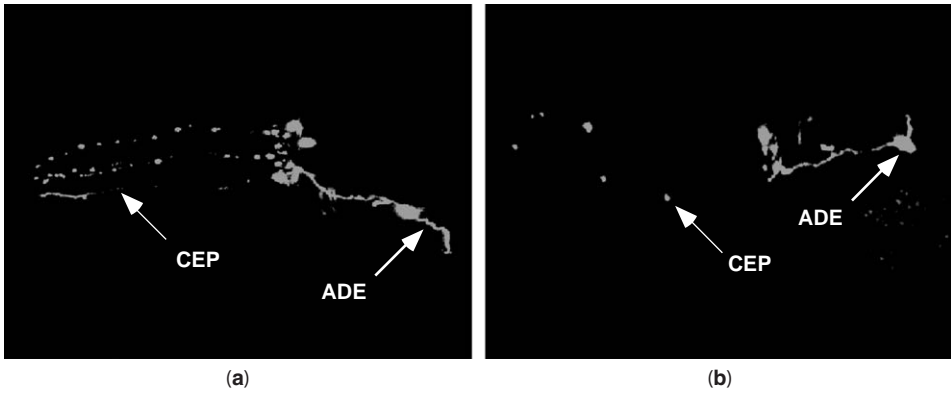


Figure 19.2 Visualization of head DA neurons in *C. elegans* following exposure to 6-OHDA: (a) early stages of cell death; (b) later stages of cell death. (See color insert.)

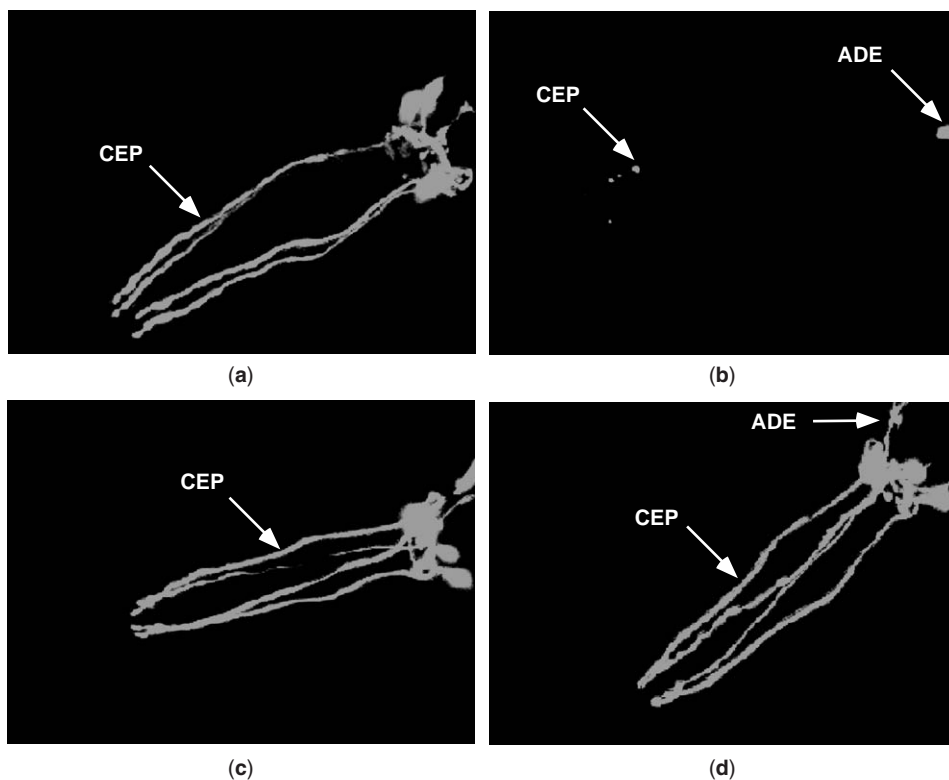


Figure 19.3 Suppression of 6-OHDA sensitivity of DA neurons in *C. elegans*: (a) *P_{dat-1}::GFP* animals exposed to vehicle; (b) *P_{dat-1}::GFP* worms exposed to 6-OHDA; (c) *P_{dat-1}::GFP, dat-1(ΔDAT-1)* worms exposed to vehicle; (d) *P_{dat-1}::GFP, dat-1* worms exposed to 6-OHDA. (Reproduced with permission from [37].) (See color insert.)

A53T α -synuclein and GFP in the DA neurons within the worm. We have found that both WT and A53T expression confers DA neuron degeneration (both cell bodies and processes) in the worm even in the absence 6-OHDA (Fig. 19.4, data not shown) and that the neurodegeneration occurs in all three subtypes of DA neurons within the hermaphrodite [48]. This effect occurs when α -synuclein is expressed either behind a pan-neuronal promoter or the DAT promoter [48]. Furthermore, motor deficits were observed when α -synuclein was expressed behind the pan-neuronal promoter, and α -synuclein-containing inclusion bodies are seen in some of the DA neurons. These results suggest that *C. elegans* could be a useful model for α -synuclein-induced pathologies.

The establishment and initial characterization of our *C. elegans* PD model also provides an opportunity to utilize genetic screens to identify novel genes involved in DA neuron cell death. For example, in one forward genetic screen we could utilize our *DAT-1::GFP* reporter line to identify molecules involved in 6-OHDA-induced neuronal death. We would first mutagenize the genome of the parental animals and then isolate the second-generation hermaphrodites (to homozygose the mutation; see [25, 26]) in which GFP is still retained in the DA neurons following exposure to 6-OHDA. Animals that have DA neurons that are insensitive to the neurotoxin could

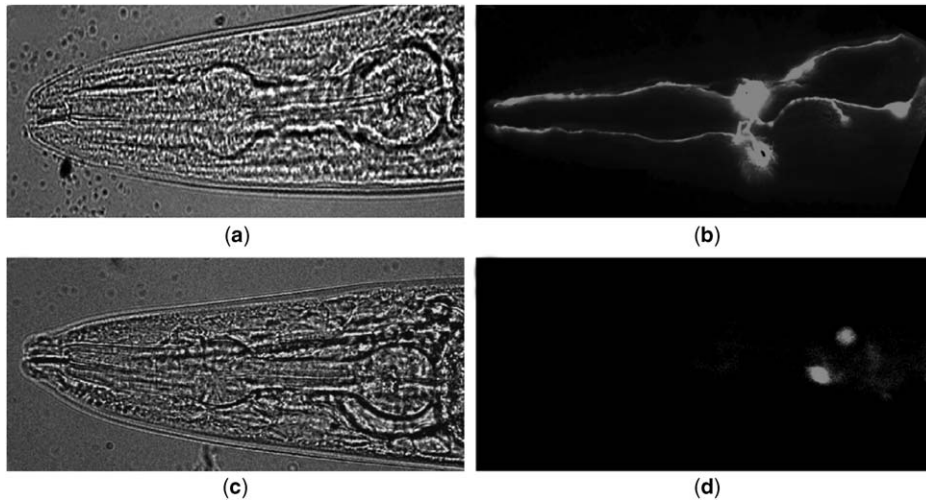


Figure 19.4 Expression of human A53T α -synuclein causes *C. elegans* DA neurons to degenerate. Anterior of adult animal in WT [(a) and (b)] shows normal complement of DA neurons, while A53T expressing animals [(c) and (d)] often show loss of cell bodies and processes. In (b) only four of the six neurons are clearly visible because of the focal plane; in (d), only the two ADE cell bodies and part of the processes are present. (See color insert.)

have mutations within DAT, DAT regulatory proteins, or proteins involved in DA neuron viability or cell death. We have implemented this forward genetic screen and have identified a number of mutants that have varying degrees of DA neuron 6-OHDA insensitivity [25, 26]. Three of these mutants contain mutations within DAT that render them completely resistant to 6-OHDA [25]. The identification of these DAT mutants provides a proof of concept that we should be able to isolate genes involved in toxin-induced cell death. We are presently mapping the other mutants to determine the role they have in DA neuron viability.

Our worm PD model also allows us to utilize a reverse-genetic approach, for example, by using RNAi. RNAi is a rapid and efficient method to decrease the expression of proteins by expressing dsRNA to a given gene or partial gene. We currently have at our disposal a library of bacteria that express RNAi molecules ($\sim 18,000$ of 20,000 genes) directed against most of the known worm genes (Medical Research Council, Cambridge). Since feeding the bacteria expressing the dsRNA to the worms is an efficient way to knock down the expression of the gene, we could envision feeding our DAT-1::GFP reporter line also expressing α -synuclein, or those exposed to 6-OHDA, the individual bacterial strains from within the library and select for animals in which the DA neurons do not degenerate. We should therefore be able to identify genes that are involved in protecting against DA neuron cell death. Unfortunately, many neurons in *C. elegans* are insensitive to RNAi. Recently, though, the identification of a RNAi-hypersensitive worm strain, *rrf-3*, greatly enhances many neurons to dsRNA [48a]. In this background we have found the DA neurons are sensitive to RNAi (Fig. 19.5), which should allow us to identify novel genes involved in PD-associated DA neuron cell death.

Feany and Bender were the first to overexpress the human WT and mutant isoforms A53T and A30P α -synuclein in fly brains, and they recapitulated many

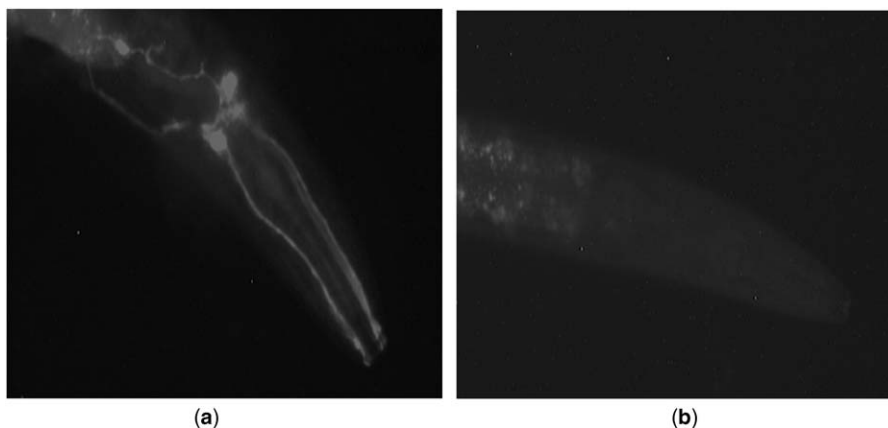


Figure 19.5 DA neurons are sensitive to RNAi in a *rrf-3* mutant background: (a) *rrf-3* animals expressing $P_{dat}::GFP$ in DA neurons grown on bacteria not expressing dsRNA for GFP; (b) *rrf-3* animals grown on bacteria that have been induced to produce dsRNA for GFP. (See color insert.)

aspects of the human disease (Fig. 19.6) [49]. With all three isoforms, they observed adult-onset loss of dopaminergic neurons, inclusions reminiscent of LBs, and loss of certain locomotor abilities to some degree.

To investigate the molecular basis of these pathologies, Scherzer and co-workers followed a functional genomics approach with the human A30P α -synuclein over-expressing strain and screened Affymetrix microarrays with RNA isolated from 1-day-old presymptomatic adults, 10-day-old mildly symptomatic adults, 30-day-old advanced symptomatic adults, and age-matched controls [50]. They found 36 genes differentially expressed in 1-day-old flies, 37 genes in 10-day-old flies, and 44 genes in 30-day-old adults, together representing 51 differentially expressed transcripts. Twenty-seven of these genes are predicted to have human orthologs. While the young flies were asymptomatic, the affected genes included many previously associated with PD pathologies such as those involved in regulation of catecholamine synthesis, lipid binding, and mitochondrial dysfunction [50]. They also found that metabolic/energy-related genes were downregulated in the 1- and 10-day-old adults but upregulated in the diseased 30-day-old transgenics. These results suggest that α -synuclein overexpression leads to downregulation of mitochondrial respiratory chain genes in young presymptomatic animals, with a progressive compensatory upregulation of other energy gene transcripts as the disease progresses [50].

Auluck and co-workers have used the overexpressing α -synuclein *Drosophila* model to identify a mechanism to prevent dopaminergic cell death [50a]. They found that human Hsp70 specifically targeted to dopaminergic neurons via a *Ddc-GAL4* driver was able to rescue dopaminergic cell loss caused by overexpression of all three α -synuclein isoforms (WT, A30P, and A53T). Hsp70 is a molecular chaperone that refolds misfolded protein. Interestingly, in the Hsp70 overexpressing tissues, levels of α -synuclein were unchanged, as were the presence and number of perinuclear inclusions [50a]. To further examine the role of Hsp70, a dominant-negative fly ortholog, Hsc4, was coexpressed with α -synuclein. Elimination of Hsc4 activity was observed to enhance the effects produced by α -synuclein overexpression. The authors propose

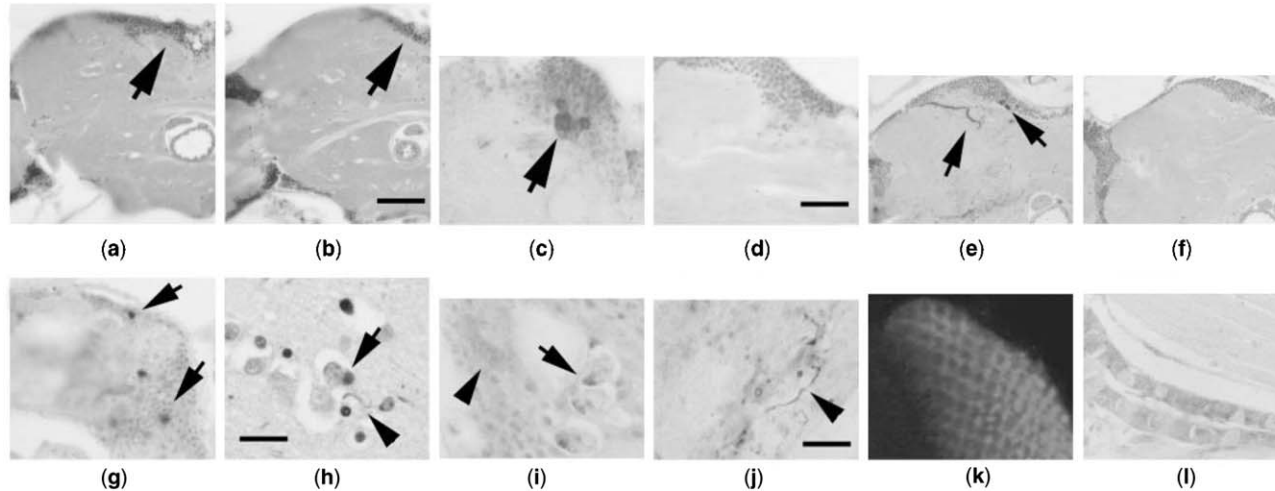


Figure 19.6 Histological and immunocytochemical analysis of α -synuclein transgenic flies. (a, b) Frontal sections of 60-day-old control fly [(a) *elav-GAL4/+*] and 60-day-old A30P α -synuclein transgenic fly [(b) *UAS-A30P α -synuclein elav-GAL4*] stained with hematoxylin and eosin. Overall brain volume, including the outer cellular cortex layer containing neuronal and glial cell bodies (arrows) and central neuropil areas, and overall architecture are preserved. (c) Thirty-day-old control fly (*elav-GAL4/+*) shows immunostaining for tyrosine hydroxylase in four to five cells in the dorsomedial cluster. (d) Thirty-day-old α -synuclein-expressing fly (*elav/+; UAS-wild-type α -synuclein/+*) shows no cell body-associated immunostaining in the same area. (e) Ten-day-old control fly expressing α -galactosidase in both cell cortex and neuronal processes (arrows) of dopaminergic neurons (*UAS-lacZ/Ddc-GAL4*). (f) Ten-day-old fly carrying in addition an α -synuclein transgene (*UAS-A30P α -synuclein/+; UAS-lacZ/Ddc-GAL4*) shows no α -galactosidase expression in the outer cellular cortex or central neuropil. (g) Immunostaining of α -synuclein inclusions in the brain from a 30-day-old transgenic fly (*UAS-A30P α -synuclein/elav-GAL4*) in the area of the subesophageal ganglia with an antibody against α -synuclein. (h) Human cortical Lewy body (arrow) from the cingulate cortex of patient with diffuse Lewy body disease, stained with an antibody against ubiquitin [same scale as (g)]. (i) Immunostaining of three α -synuclein inclusions in the brain of a young adult fly (one day posteclosion; *Ddc-GAL4/UAS-wild-type α -synuclein*) showing irregularity of selected inclusions (arrow) and diffuse immunoreactivity in a larger neuron (arrowhead). (j) Neuritic pathology consisting of α -synuclein immunoreactive thread (arrowhead) and grain-like structures. Sixty-day-old fly (*UAS-A30P α -synuclein/elav-GAL4*). Compare with abnormal neurite in the cingulate cortex of a Lewy body disease patient [(h) arrowhead]. (k) Immunofluorescence staining of eye imaginal disc from wandering third instar larva (*UAS-A53 T α -synuclein/+; gmr-GAL4/+*) with antibody against α -synuclein showing no inclusions. (l) Diffuse cytoplasmic α -synuclein immunoreactivity in the adult gut from a 30-day-old fly (*UAS-A53 T α -synuclein/+; e29c-GAL4/+*). Scale bars: (a,b,e,f) 30 μ m; (c,d,g,h) 10 μ m; (i,j) 5 μ m. (Reproduced with permission from [49].) (See color insert.)

two possible roles for chaperones in α -synuclein toxicity: Endogenous chaperones may normally provide protection against toxicity by delaying the onset of degeneration and accumulated α -synuclein may interfere with endogenous chaperone activity [50a].

An inherited form of PD, autosomal recessive juvenile PD, involves the *parkin* gene, which encodes for an E3 ubiquitin ligase, one of a number of proteins that ubiquitinate substrate proteins and mark them for proteasome degradation. Many of parkin's substrates are associated with α -synuclein and α -synuclein-interacting proteins. Loss-of-parkin function may result in the accumulation of specific proteins to toxic levels that ultimately lead to neuronal degeneration and cell death. Heywood and Staveley identified the *Drosophila* ortholog of parkin and overexpressed it in transgenic animals [51]. Parkin overexpression alone in dopaminergic neurons or the eye had no overt effects on neuronal degeneration, locomotion, or life span. However, when parkin was overexpressed in combination with α -synuclein, the effects of α -synuclein overexpression (neurodegeneration, locomotor deficits, reduced life span) were significantly reduced [51]. The authors put forth a model in which parkin binds to glycosylated α -synuclein via a ubiquitin-like (UBL) domain/unique parkin domain, and to E2 ubiquitin via really interesting new gene (RING) finger domains. E2 ubiquitin ubiquitinates α -synuclein, and targets to the proteasome, which binds to parkin's UBL domain [51]. In the absence of parkin, α -synuclein accumulates to toxic levels, presumably leading to PD-like pathologies.

A parkin homolog has been identified in *C. elegans* (K08E3.7). It is approximately 29% identical to the human gene. Preliminary results indicate that a putative knockout of parkin is almost two fold more sensitive to 6-OHDA relative to WT animals (M. Marvanova and R. Nass, data not shown).

Some transition metals have also been associated with PD, and our preliminary experiments suggest that at least in worm the DA neurons can be sensitive. Several epidemiological studies suggest a significant correlation of chronic Mn^{2+} exposures with the propensity to develop PD. Indeed, the strongest correlation between heavy-metal exposure and increased susceptibility to PD was made in Mn^{2+} -exposed individuals [52, 53]. It is also noteworthy in a study by Racette et al [54] that parkinsonism in welders is distinguished clinically only by age at onset, suggesting welding (an occupation with potentially high exposure to Mn^{2+}) may be a risk factor for PD. Individuals with occupational exposure to Mn^{2+} for greater than 20 years have an increased probability to develop PD [53]. Aggregation and fibrillation of α -synuclein is also dramatically accelerated in vitro in the presence of Mn^{2+} [55, 56]. Mn^{2+} also increases the expression of *parkin* gene in vitro and causes aggregation of the protein in DA cells [57]. Overexpression of parkin also protects DA cells from Mn^{2+} -induced cell death, suggesting that parkin could play a role in Mn^{2+} -induced cell death in vivo [57]. Epidemiological studies have also established an increased risk for PD in association with occupational exposure to Fe^{2+} and Al^{3+} , especially greater than 30 years [55, 56, 58, 59]. In several studies, postmortem analysis of brains from patients with PD showed that there was a considerable increase in total Fe^{2+} and Al^{3+} content in the substantia nigra relative to controls. Also, unilateral injection of Fe^{2+} into the substantia nigra of rats results in a 95% decrease in striatal DA, supporting the hypothesis that Fe^{2+} could play a role in DA neuron death in PD. The toxic aggregation of α -synuclein was also found to be much more rapidly accelerated in vitro when combined with Fe^{2+} or Al^{3+} , suggesting that these metals may contribute to PD. Our preliminary studies have also suggested that these heavy

metals can confer DA neuron cell death in *C. elegans* and that this system could also be used to explore the mechanism of metal-induced DA neurodegeneration (R. Nass, M. Marvanova, M. Fullard, data not shown).

Chronic exposure to rotenone in rats has also been shown to produce PD-like symptoms and pathologies. Rotenone is an inhibitor of mitochondrial respiratory chain complex I. Recently, Coulom and Birman have shown that chronic sublethal exposure of flies to rotenone produces selective dopaminergic cell death and locomotor deficits similar to that seen in another PD fly model [60]. A significant difference was that rotenone produced a much more severe dopaminergic cell loss than the α -synuclein model and there were no detectable perinuclear inclusions induced by rotenone. The authors note that this observation is consistent with mutations in parkin, in which dopaminergic cell loss is produced without the formation of Lewy bodies. This particular *Drosophila* system provides a model to study oxidative stress as it relates to neurodegeneration. The fly parkin mutants also exhibit mitochondrial pathology, muscle degeneration, and male sterility and are sensitive to heavy metals, which at least in part is suggestive of what is observed in sporadic PD.

19.6 MODELING TRINUCLEOTIDE REPEATS OR HUNTINGTON'S DISEASE

Huntington's disease (HD) is a progressive neurodegenerative disease that is caused by the CAG repeat encoding the poly (Q) tract in the N-terminus of the protein huntingtin [61, 62]. There are at least nine other human similar disorders, including the X-linked motoneuron disease spinobulbar muscular atrophy (SMBA) that affects the androgen receptor. Neuron degeneration occurs when the poly-Q repeat can exceed 35 or greater, and generally longer repeats cause an earlier date of onset of the disease. These abnormal proteins form aggregates and can interact inappropriately with other cellular factors that may ultimately lead to cell death. The molecular mechanisms underlying these disorders are not well understood. HD and other poly-Q diseases all share dominance, late onset, and progressive neurodegeneration.

Caenorhabditis elegans has been utilized to evaluate the role the poly Q-repeats have on the neurodegeneration and to screen for novel proteins that may be involved in the pathogenesis. Overexpression of the protein fragment in the worm recapitulates the progressive loss of neurons in which the protein is expressed. Faber et al. have expressed different lengths of the poly-Q protein behind the *C. elegans osm-10* promoter, resulting in the expression of the aggregated protein in eight sensory neurons [63]. These neurons are exposed to the environment and normally take up a fluorescent dye. Dye uptake is decreased when there is expression of the poly-Q protein in these neurons, and there is greater protein aggregation with increased expression. Furthermore, cell death is positively correlated with the poly-Q repeat lengths.

Since CAG repeats can confer neuronal death in *C. elegans*, RNAi screening can be utilized to identify suppressors or enhancers of poly-Q-induced cell death. Nollen et al. expressed Q residues fused to the yellow fluorescent protein (YFP) in muscle cells to identify genes that prevent protein aggregate formation [64]. Transgenic animals expressing YFP fused to a low copy number of poly-Q displayed a diffused

staining pattern, while those expressing Q35 showed a punctate or aggregate formation in adulthood. Growth of the worms on bacteria expressing dsRNA to knock down endogenous *C. elegans* proteins identified 186 proteins that result in premature appearance of the aggregates [64]. These genes include proteins involved in protein synthesis, folding, and transport as well as degradation. Surprisingly, the screen identified a number of proteins involved in RNA synthesis and processing, possibly by increasing the influx of misfolded proteins or by somehow disrupting the overall equilibrium of production and degradation of proteins involved in the regulation of the proteasome. This screen demonstrates the power of RNAi screening in *C. elegans* to identify novel proteins that could be involved in human polyglutamine disorders.

Jackson and co-workers have also created a *Drosophila* model that resembles many aspects of HD. Transgenic flies that overexpresses the first 171 residues of human huntingtin (htt) containing a 120-residue polyglutamine region within photoreceptors of the fly eye show profound retinal degeneration [65]. Expression of the Gln₁₂₀ htt, but not the WT Gln₂₃ htt, produces nuclear inclusions and age-dependent degeneration of photoreceptor cells with the severity correlating to the length of the polyglutamine expansion. Ravikumar and co-workers were able to significantly slow retinal degeneration in this model by treating flies with rapamycin [66]. Experiments in mammalian systems indicate that the mutant huntingtin inappropriately interacts with the molecular target of rapamycin (mTOR) and inhibits its function. Because mTOR is involved in many key cellular functions ranging from protein synthesis to cell cycle control, they speculate that this inhibition may ultimately be a contributing factor to cell death [66]. Rapamycin impairs mTOR function, which leads to an induction of autophagy. The authors speculate that the observed increase in autophagy increases degradation and elimination of the mutant htt, thereby protecting against neurodegeneration [66].

An additional poly-Q model in the fly was created by Warrick and co-workers [67]. They expressed the C-terminus of ataxin-3, the gene implicated in spinocerebellar ataxia type 3 (SCA3/MJD), containing either Gln₂₇ (control) or Gln₇₈ (mutant) within the eye of the fly. Targeted expression of the mutant, but not the control, protein to photoreceptors within the eye produced nuclear inclusions and late-onset, progressive degeneration of the retina that led to gross morphological changes of the eye [67]. Similar to the results of Jackson et al. [65], they found that the severity of the degenerative phenotype depended on the length of the poly-Q repeat region. Targeted expression of the mutant, but not control, protein to the central nervous system (CNS) produced early adult viability at low to moderate levels of expression and embryonic lethality at high levels of expression. The authors note that in humans poly-Q diseases only cause degeneration of neurons. To see if this was also true in flies, they expressed mutant ataxin-3 in muscle and epithelial cells. Targeting of the mutant protein muscle tissues produced lethality at the larval stage, but targeting to epithelial cells via the *dpp* promoter had no effect on viability or morphology. Interestingly, coexpression of the antiapoptotic baculoviral gene *P35* within the eye significantly attenuates the toxic effects of the mutant ataxin-3 [67].

Kretzchmar and co-workers have investigated the effects of expressing the control or mutant ataxin-3 fragment in either neurons or glial cells on fly behaviors and viability [68]. They found that expression of both forms of ataxin-3 in both neural and glial tissues reduced the life span of flies, although the mutant form produced

death much quicker. Similar to the effects seen in neurons, glial cells accumulated nuclear inclusions and degenerated over time, with the Gln₇₈ ataxin-3 fragment producing the strongest phenotype [68]. Two behaviors were analyzed, fast phototaxis and walking behavior (Buridan's paradigm). Over the first 20 days of adult life, flies expressing both forms of the ataxin-3 fragment showed age-dependent loss of phototaxis. In walking behavior, however, there was no observable deficit over the 10 days tested, suggesting that the poly-Q affected brain regions do not significantly contribute to this walking behavior [68]. Based upon their results, the authors draw particular attention to the potential role of glial cells in the etiology of poly-Q diseases and note that the use of behavioral assays such as fast phototaxis will be useful for performing modifier screens to identify genetic factors involved in the pathogenesis of poly-Q diseases.

Many studies in mammalian systems have demonstrated sequestration of cyclic adenosine monophosphate response element binding (CREB) protein (CBP) by poly-Q aggregates, and this phenomenon was investigated in the fly by Taylor and co-workers [69]. They hypothesized that sequestration of CBP, a histone acetyltransferase, would reduce normal histone acetylation and give rise to altered transcription patterns, which may be an underlying cause of the pathogenesis of poly-Q diseases. To test the role of CBP, they expressed Gln₁₂₇ in the eye of the fly to produce age-dependent degeneration, then induced overexpression of the gene encoding endogenous dCBP, *nejire*. Overexpression of dCBP completely rescued the degenerative phenotype of Gln₁₂₇ within the eye. Furthermore, they observed that disruption of *nejire* exacerbated the degenerative phenotype of Gln₁₂₇ expression in the eye [69]. Surprisingly, while the amount of cellular Gln₁₂₇ was unchanged, poly-Q aggregates were virtually absent in the dCBP rescued flies [69]. Extracts from Gln₁₂₇ fly eyes showed significant reductions in histone acetyltransferase activity, which was rescued with dCBP overexpression [69]. Significantly, microarray studies using RNA from one- to two-day-old adult flies in the process of Gln₁₂₇-induced degeneration showed altered expression of a small number of genes that was partially restored by dCBP overexpression. The authors propose a model in which poly-Q monomers aggregate over time, leading to sequestration of CBP and aberrant histone acetylation. These conditions result in altered transcription and subsequent neuronal dysfunction and death [69]. In summary, *Drosophila* studies of poly-Q disease mechanisms have identified possible new pathogenic mechanisms (i.e., glial cells) and have pointed in novel directions for the development of therapeutics, including rapamycin, expression of antiapoptotic genes like *P35*, and restoration of CBP activity.

19.7 MODELING ALZHEIMERS'S DISEASE

Alzheimer's disease (AD) involves the progressive loss of memory and recognition and is the most prevalent neurodegenerative disease in the United States. Pathologically, the disease causes the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles in the CNS and the degeneration of neurons in the forebrain and hippocampus [70].

Although the molecular mechanisms of AD have not been elucidated, it is believed that the major component of the β -amyloid plaques, A β 1-42, which is cleaved from

the precursor protein APP, significantly contributes to the pathogenesis [71]. Mutation in APP has been associated with some familial early-onset AD overproduction of A β through the activity of β - or γ -secretase and has contributed to plaque deposition [71, 72]. Normally, APP is processed into a number of peptides either by β -secretase activity of the β -site APP-cleaving enzyme (BACE) or by γ -secretase activity of a large complex containing presenilins 1 or 2 (PS1, PS2), nicastrin, aph-1, and pen2. A number of familial AD mutations are changes in presenilins that lead to altered APP processing and to increased ratios of A β_{42} to A β_{total} [73, 74].

Caenorhabditis elegans contains a human homolog to APP, called apl-1, though the homologous sequence to A β_{1-42} is absent [75, 76]. The absence of this part of the protein, however, fortuitously allows for the examination of the molecular basis of the pathogenesis in transgenic animals overexpressing the human form of A β without potentially complicating interpretations due to the interference of the endogenous protein. Interestingly, though, RNAi knockdown of the worm APP results in an uncoordinated phenotype, and a genetic deletion results in lethality [76, 77]. Significantly, overexpression of human A β_{1-42} in the muscle wall of the worm causes progressive paralysis, amyloid-like deposits in vivo, and a decrease in life span, consistent with the idea that A β plays a significant role in conferring the pathological changes involved in AD [76, 78, 78a].

Caenorhabditis elegans has a presenilin homolog, *sel-12* [79]. Mutations in *sel-12* suppress mutations in the *C. elegans* variant of the notch pathway, suggesting as in the human AD that these genes play a role in presenilin-dependent proteolysis. These studies further highlighted the role of abnormal Notch function in AD pathology.

The neurofibrillar aggregates AD contain the microtubule binding protein tau [80]. Mutations within tau have been associated with frontotemporal dementia with Parkinson's (FTDP-17) [81]. Deletion of the worm homolog of tau, *ptl-1*, does not result in any apparent phenotype, although human expression of tau or the FTDP-17 mutant tau in worm causes uncoordinated movement, suggesting AD-associated disruptions in normal neurotransmission [82]. The authors also showed that the FTDP-17 was more toxic than Wt, that there was age-dependent accumulation of insoluble tau, and that the neurodegeneration was progressive. These results suggest that this model could be utilized to look for interacting molecules involved in tau-induced neurodegeneration.

There have been many advances using *Drosophila* to model certain of the pathological changes associated with AD. Although *Drosophila* endogenously expresses an ortholog of APP (APPL), the Ab domain is not conserved. The γ -secretase pathway and components are also conserved in the fly, but there is little to no endogenous β -secretase activity. APPL null mutants show subtle behavioral deficits, and these deficits can be partially rescued by human APP [83].

One model to investigate the pathological effects of accumulated AB has been to overexpress both A β_{40} and A β_{42} within flies using the GAL4/UAS system [84, 85]. Expression of A β_{42} , but not A β_{40} , in the eye of the fly produces retinal degeneration and accumulation of plaques with age (Fig. 19.7) [84]. Overexpression of A β_{42} in the entire CNS results in a significant reduction of life span, whereas overexpression of A β_{40} does not [84, 85]. Although it would appear that A β_{40} is not contributing to observable pathological processes, it does seem to be involved in behavior. Flies overexpressing A β_{40} , as well as those overexpressing A β_{42} , in the CNS show

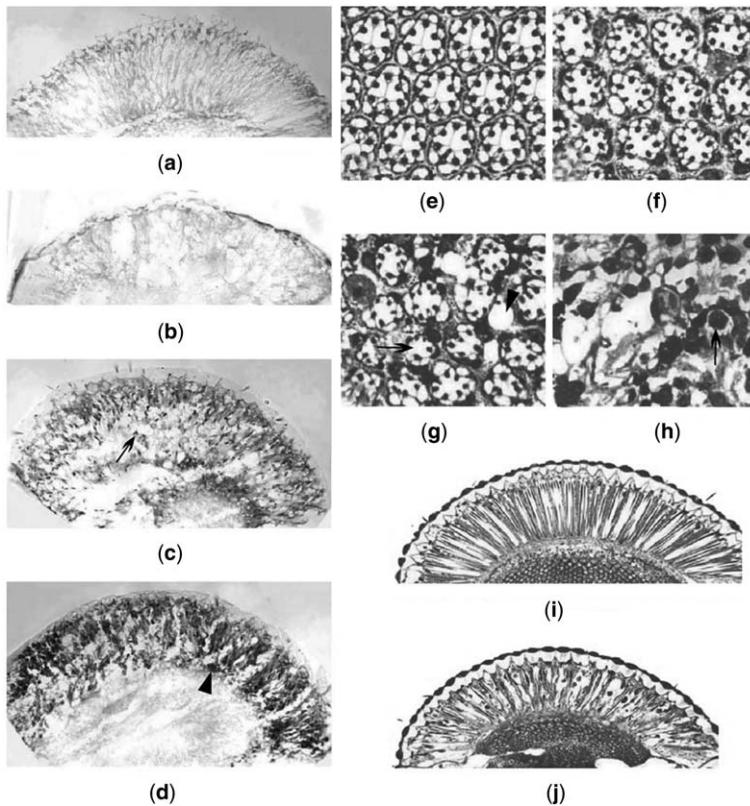


Figure 19.7 Levels of $A\beta_{42}$ accumulation correlate with eye disorganization. (a–d) Horizontal 12- μ m cryosections of (4)- to (5)-day old adult eyes immunolabeled with 6E10 mAb and visualized with a biotinylated secondary antibody and diaminobenzidine. (a) Normal retina of pGMR-1 flies, probed with 6E10 and secondary antibody. (b) Disrupted retina in the absence of $A\beta_{42}$ staining in GMR- $A\beta_{42}$ K10 homozygous flies, probed with secondary antibody only. (c) Punctate $A\beta_{42}$ staining in GMR- $A\beta_{42}$ K3 homozygous eyes (arrow), probed with 6E10 and secondary antibody. (d) Larger aggregates of $A\beta_{42}$ staining in GMR- $A\beta_{42}$ K10 homozygous eyes (arrowhead), probed with 6E10 and secondary. (e–j) Plastic sections (1.5 μ m) of (4)- to (5)-day-old adult eyes stained with toluidine blue, highlighting ommatidial organization: (e–h) tangential sections; (i, j) horizontal sections. (e) Regular array of ommatidia in pGMR1 eyes. (f) Eyes of GMR- $A\beta_{42}$ K2 heterozygous flies show some photoreceptor abnormalities even though no phenotype is visible at the gross morphological level. (g) Eyes of GMR- $A\beta_{42}$ K3 homozygous flies show missing photoreceptors (arrow) and gaps in the tissue (arrowhead). (h) Eyes of GMR- $A\beta_{42}$ K52 homozygous; GMR- $A\beta_{42}$ K53 homozygous flies show almost a complete loss of ommatidia with occasional residual photoreceptors (arrow) and large tissue gaps. (i) Long, intact photoreceptor cells in pGMR1 eyes. (j) Disrupted photoreceptor cells in eyes of GMR- $A\beta_{42}$ K3 homozygous flies. Dense-staining, dying photoreceptors are present in all $A\beta_{42}$ -expressing eyes. (Reproduced with permission from [84].) (See color insert.)

age-dependent olfactory learning deficits. The learning difficulties associated with $A\beta_{40}$, however, begin to appear at a later age.

Because the $A\beta_{40}$ learning problems are not associated with neurodegeneration, Lijima and co-workers speculated that neuronal dysfunction and degeneration may

be mediated by different mechanisms [85]. To identify potential modifiers, Finelli and co-workers screened approximately 2000 expression P (EP strains) and identified a small collection that altered the $A\beta_{42}$ degenerative eye phenotype [84]. One of these modifier EP strains was shown to have upregulated *neprilysin 2* (*nep2*) expression. NEP2 is a soluble endopeptidase whose expression is normally restricted to renal tubules and testis [86]. In flies overexpressing $A\beta_{42}$ within the eye, NEP2 expression reduces levels of $A\beta_{42}$, and in flies overexpressing $A\beta_{42}$ in the CNS, NEP2 rescues the longevity phenotype [84].

Because flies have no endogenous β -secretase activity and *Drosophila* APPL has no Ab domain, Greeve and co-workers created transgenics expressing both the human BACE and APP genes [87]. Coexpression of these two proteins resulted in processing of APP at the β site. APP and BACE expressed in the eye produced severe, age-dependent degeneration of photoreceptor cells and their axon projections and deposition of β -amyloid plaques. Interestingly, neural degeneration preceded the appearance of plaques. Within this model system, the activity of γ -secretase was modulated by coexpressing various presenilin alleles, including three human Familial Alzheimer's Disease (FAD) mutants. Loss of function presenilin alleles suppressed degeneration, whereas coexpression of the FAD mutants enhanced degeneration [87]. These results indicate that γ -secretase activity is crucial to the retinal degeneration phenotype. Expression of APP and BACE ectopically in flies via actin-GAL4 produced semilethality (reduced eclosion rate), ectopic veins in the wing, and a reduced adult life span [87]. Each of these phenotypes could be partially rescued by growing flies on food to which BACE or γ -secretase inhibitors had been added. The authors of this study note that the wing vein phenotype and subsequent pharmacological rescue may represent a novel screening method for factors influencing APP processing.

19.8 PERSPECTIVES AND FUTURE DIRECTIONS

The model organisms *C. elegans* and *D. melanogaster* provide remarkable opportunities to identify and characterize potential human disease genes and proteins in vivo. The high similarities on the molecular level between invertebrates and humans suggest that the paradigms discovered using these systems are highly relevant to mammalian neurodegenerative diseases. As briefly discussed in this chapter, mammalian proteins associated with a number of neurodegenerative diseases can complement their endogenous function in invertebrate systems and recapitulate many aspects of their pathology. The existence of a large number of worm and fly strains should greatly facilitate the further elucidation of the role that these proteins play in biology and disease. The amendability of both systems to forward and reverse genetic screens will likely help to identify novel molecules and pathways involved in neurodegeneration. These invertebrates can also be utilized to screen and identify environmental agents, such as pesticides, fungicides, or other xenobiotics, that could cause or contribute to susceptibility to disease.

The sensitivity of invertebrate models also provides the opportunity for medium- or high-throughput screens (HTSs) to identify drug leads and novel therapeutics that could protect against cell death. Suppressor screens could then identify the drug's molecular target or define the pathway. These experiments can be performed without

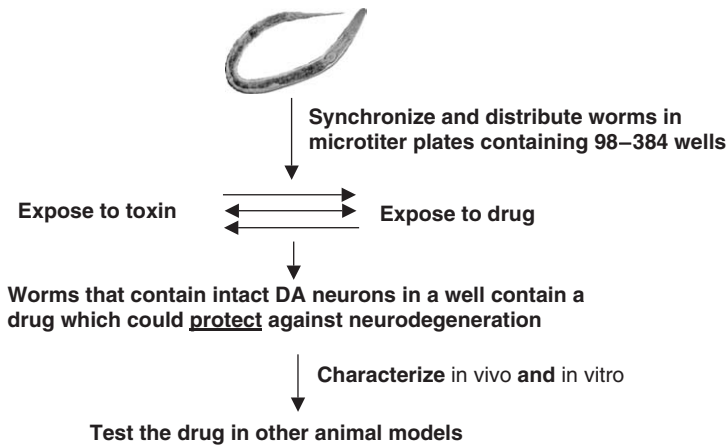


Figure 19.8 Proposed high-throughput screen for compounds that protect against DA neuron cell death in *C. elegans*. Compound that inhibits loss of GFP in DA neurons protects against DA neurodegeneration and warrants further assays in secondary screens.

any a priori knowledge of the molecular basis of the drug's protection. For example, since *C. elegans* can easily be grown in liquid medium, we could expose our DA neuron reporter line expressing α -synuclein to drug libraries in 96-well microtiter plates. The plate can then be analyzed in a fluorescent plate reader. Those wells in which the DA neurons still fluoresce contain a compound that has the potential to be neuroprotective in mammalian systems. Our preliminary studies indicate that the DA neuron fluorescence can easily be detected in a platereader. A similar screen can also be incorporated using known PD-associated neurotoxins (Fig. 19.8). Once a therapeutic has been identified, the power of *C. elegans* provides a unique opportunity to do suppressor chemical screens to identify the molecules and interacting partners involved in the compound-mediated protection of the DA neurons. We would simply mutate the animals and screen for worms in which the DA neurons are *not* protected by the drug. Using standard *C. elegans* genetics and molecular biology, we could then quickly identify the drug targets and pathways involved in the drug's efficacy.

These exciting studies can provide new insight into the molecular basis of human neurodegenerative diseases and identify effective therapeutic targets. It is very likely that invertebrates will play an integral role in the future of pharmacology, toxicology, and human drug discovery.

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MYELIN LIPIDS AND PROTEINS: STRUCTURE, FUNCTION, AND ROLES IN NEUROLOGICAL DISORDERS

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20.1	Structure of Myelin	592
20.2	Composition of Myelin	592
20.2.1	Lipids of Myelin	594
20.2.2	Proteins of CNS Myelin	596
20.2.2.1	Proteolipid Protein	596
20.2.2.2	Myelin Basic Protein	598
20.2.3	Proteins of PNS Myelin	599
20.2.3.1	Protein Zero	599
20.2.3.2	Peripheral Myelin Protein-22	601
20.2.3.3	Myelin Basic Protein	602
20.2.3.4	Protein 2	603
20.2.4	Other Proteins in Myelin and Myelin-Related Membranes	603
20.2.4.1	2',3'-Cyclic Nucleotide 3'-Phosphodiesterase	603
20.2.4.2	Glycoproteins Present in Small Amounts in Internodes of Myelin Sheaths	604
20.2.4.3	Tetraspan Proteins	607
20.2.4.4	Other Paranodal Proteins	609
20.2.4.5	Enzymes Associated with Myelin	609
20.2.4.6	Receptors Associated with Myelin	610
20.3	Disorders of Myelin	611
20.3.1	Pathology of Myelinated Axons Related to Disruption of Axon–Glia Interactions	611
20.3.2	Autoimmune Demyelinating Diseases	611
20.3.2.1	Experimental Allergic Encephalomyelitis	611
20.3.2.2	Multiple Sclerosis	612
20.3.2.3	Autoimmune Demyelinating Disorders of PNS	613
20.4	Summary and Conclusions	615
	Acknowledgment	616
	References	616

Large axons in the peripheral and central nervous systems (PNS, CNS) of vertebrates are surrounded by myelin sheaths. These sheaths facilitate rapid conduction of action potentials and also conserve the amount of energy required for maintaining ion gradients [1]. The objective of this chapter is to provide an overview of myelin and myelin-forming cells that will serve as a foundation for other chapters in this section that cover diseases affecting myelin. The structure and function of proteins and lipids that are important for the formation and maintenance of myelin sheaths will be summarized. In addition, their roles as target antigens in the immunological aspects of demyelinating diseases will be discussed.

20.1 STRUCTURE OF MYELIN

In the PNS, myelin sheaths are formed as spiraled extensions of Schwann cell plasma membranes around the axons which are compacted to form the typical layered structure of mature myelin (Figs. 20.1a and 20.2a). Each Schwann cell forms a single segment of myelin around an axon. These segments of compact myelin are called internodes, because they are separated by specialized structures called nodes where ion fluxes across the axonal membrane generate action potentials (Fig. 20.1b). In the CNS, the myelin-forming cells are oligodendrocytes, and the myelin sheaths are formed similarly as spiraled extensions of their plasma membranes (Fig. 20.1c). However, the relationship of oligodendrocytes to mature myelin sheaths is more complex, because each oligodendrocyte can send out more than one process, each one of which forms a separate internode of myelin around different axons.

The ultrastructure of myelin at the electron microscope level is characterized by alternating major dense and intraperiod lines that form when the spiraled cell surface membranes compact (Figs. 20.1 and 20.2). The major dense lines in compact myelin correspond to the fused cytoplasmic surfaces of the myelin-forming cells. The less dark, or intraperiod, lines represent the closely apposed extracellular surfaces of the glial cell membranes, which can be resolved as a double line in high-resolution electron micrographs. Furthermore, although the membranes extending from the perikarya of myelin-forming cells to compact myelin are continuous, there are many specializations within the membranes that exhibit variability in terms of structure, function, and biochemical composition. Besides the compact myelin itself, the specialized domains include the outer (abaxonal) and inner (periaxonal or adaxonal) membranes of the sheath, lateral loops in the paranodal regions, and incisures (Figs. 20.1b,c). When considering function or pathological processes involving myelin, it is important to distinguish the constituents of compact myelin from those of these other specialized membranes in the sheaths. A more detailed description of the structure, function, and biochemistry of myelin and its relationship to myelin-forming glia is available elsewhere [1].

20.2 COMPOSITION OF MYELIN

A novel characteristic of compact myelin in both the PNS and CNS is the high proportion of lipid, which accounts for 70–85% of the dry weight [1]. Therefore, the proteins only account for 15–30%. By comparison, most biological membranes have

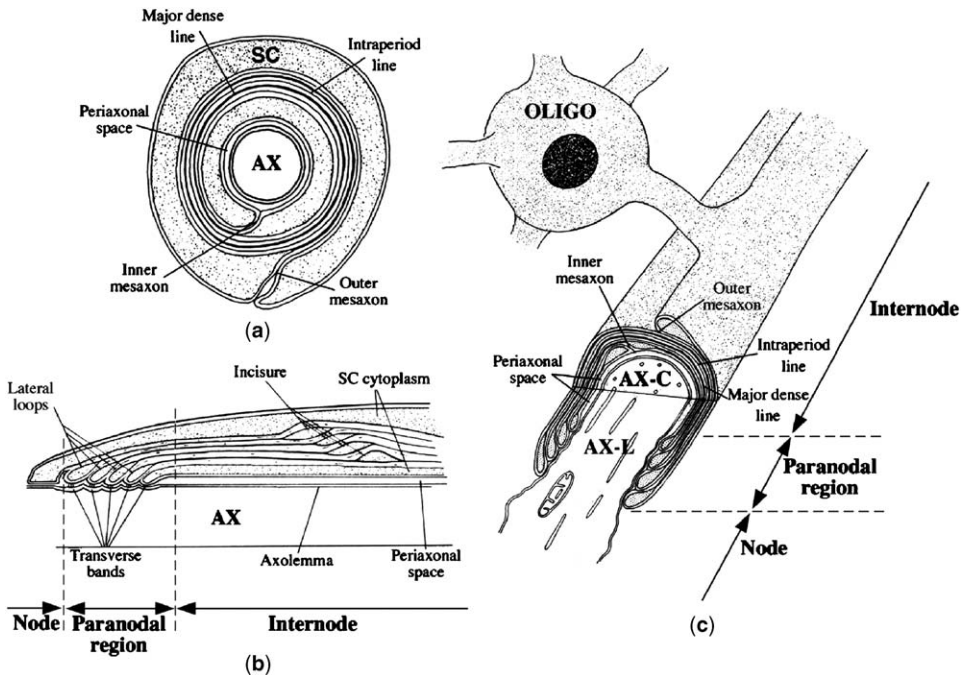


Figure 20.1 Relationship of myelin-forming glia to compact myelin. Only a few layers of compact myelin are shown for simplification, but mature sheaths have many layers, as shown in the micrographs in Fig. 20.2. (a) In PNS, the Schwann cell (SC) surrounds the axon (AX) and the mesaxon where the SC plasma membrane comes in contact with itself forms a spiral around the axon and is tightly layered to form the compact myelin. The major dense lines represent the apposition of the cytoplasmic surfaces of the glial plasma membranes, whereas the intraperiod lines represent the apposition of the extracellular surfaces. Rings of SC cytoplasm (speckled areas) are retained inside and outside the compact myelin even in mature sheaths. The periaxonal space is the extracellular gap between the inner periaxonal SC membrane and the axonal surface membrane. (b) Myelinated axon in PNS from longitudinal perspective. At the end of each segment of compact myelin (internode) is a node of Ranvier, which is not covered by myelin. Between the node and the internode is the paranodal region with highly specialized structures, where the lateral loops of the Schwann cells form tight junctions (transverse bands) with the axon. The lateral loops and Schmidt–Lantermann incisures are regions containing SC cytoplasm where the membranes are not compacted. (c) CNS myelin formed in similar way by spiraling of plasma membrane of oligodendrocyte (OLIGO). Myelinated axon is shown partly in cross section (AX-C) and partly longitudinally (AX-L). Whereas each SC forms only one internode of myelin, each oligodendrocyte sends out multiple processes each one of which forms an internode of myelin. [From R. H. Quarles (2002). *Cell. Mol. Life Sci.* 59, 1851–1871. Reproduced with permission of Birkhauser Verlag AG.]

a higher ratio of proteins to lipids. Proteins and lipids are asymmetrically distributed in the lipid bilayer, with only partial asymmetry of the lipids. The proposed molecular architecture of the layered membranes of compact myelin fits such a concept (Fig. 20.3). Models of compact myelin are based on data from electron microscopy, immunostaining, X-ray diffraction, surface probe studies, structural

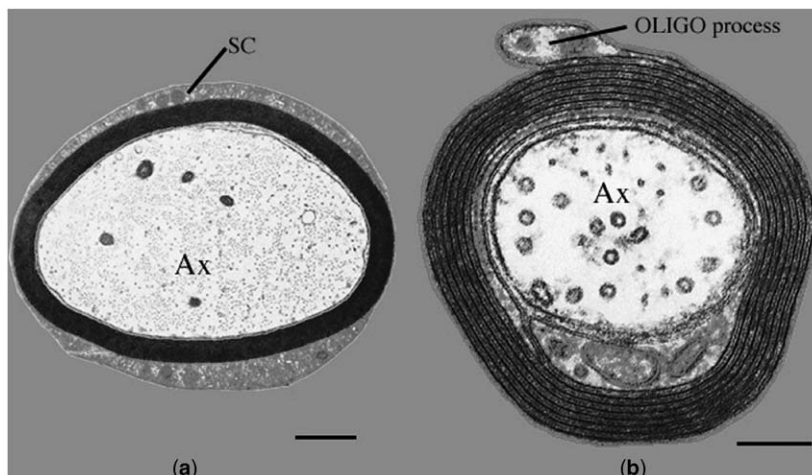


Figure 20.2 Color-coded electron micrographs of PNS (a) and CNS (b) myelin sheaths showing locations of various proteins. The PNS sheath is much larger than the CNS sheath, so the magnification in (b) is over 10-fold greater than in (a). [Scale bars: (a) 1.0 μm ; (b) 0.1 μm .] At the higher magnification in (b) the characteristic alternating major dense and intraperiod lines are visible. The cytoplasm of the Schwann cell (SC) and oligodendrocyte (OLIGO) is highlighted in green, and the end of an oligodendrocyte process leading to the myelin sheath is labeled. Protein zero (P0) and peripheral myelin protein-22 (PMP-22) are components of the layered compact myelin (gray) in the PNS (a). Neither of these proteins is in compact CNS myelin, in which the major integral membrane protein is proteolipid protein (PLP). Myelin basic protein (MBP) is a prominent component of the compact myelin in both PNS and CNS sheaths. Myelin-associated glycoprotein (MAG, yellow) is localized in periaxonal Schwann cell and oligodendroglial membranes of both PNS and CNS myelin sheaths, where it projects into the periaxonal space and participates in glia-axon interactions. Myelin oligodendrocyte glycoprotein (MOG, red) is specific to the CNS and is localized on the outside surfaces of myelin sheaths and oligodendrocytes, where it is accessible to interact with components of the extracellular environment. [From R. H. Quarles (2002). *Cell. Mol. Life Sci.* 59, 1851–1871. Reproduced with permission of Birkhauser Verlag AG.] (See color insert.)

abnormalities in mutant mice, correlations between structure and composition in various species, and predictions of protein structure from sequencing information [2].

20.2.1 Lipids of Myelin

While there are no absolutely “myelin-specific” lipids, galactosyl ceramide (cerebroside) is the most characteristic of myelin [1]. About one-fifth of the total galactolipid in myelin is sulfatide, in which the galactose of cerebroside is sulfated on the 3-hydroxyl moiety. Presumably, the glycolipids in myelin, as in other membranes, are preferentially localized on the extracellular membrane face at the intraperiod line. In addition to their localization in compact myelin, both cerebroside and sulfatide are expressed on the surface of myelin-forming glia. Mice lacking a key enzyme in cerebroside synthesis, uridine diphosphate (UDP)-galactose:ceramide galactosyl-transferase, express no cerebroside or sulfatide. Although myelin is formed relatively normally in these mice, there are subtle structural alterations in the myelin sheaths

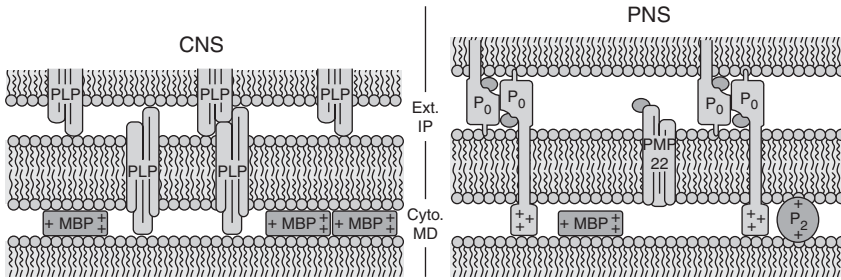


Figure 20.3 Diagrammatic representation of current concepts of molecular organization of compact CNS and PNS myelin. The apposition of the extracellular (Ext) surfaces of the oligodendrocyte or Schwann cell membranes to form the intraperiod (IP) line is shown in the upper part. The apposition of the cytoplasmic (Cyto) surfaces of the membranes of the myelin-forming cells to form the major dense (MD) line is shown in the lower part. The width of the lipid bilayers and the spacing of the intraperiod and major dense lines are proportional to those determined by X-ray diffraction [2]. The dark oval structures on P0 and PMP represent the single oligosaccharide moieties on each of these glycoproteins. The blip at the apex of P0 represents the tryptophan residue, which X-ray analysis suggests may interact with the apposing bilayer, but the expected tetramerization of P0 is not shown for diagrammatic simplification. Although PLP molecules may exhibit homophilic interactions as suggested at one position in the figure, there is no strong experimental evidence to support this as in the case of P0. 2',3'-Cyclic nucleotide 3' phosphodiesterase (CNP), MAG, and other quantitatively minor proteins of isolated myelin are not included because they probably do not play a major structural role in most of the compact myelin. In fact, many of them are localized selectively in regions of myelin sheaths distinct from the compact myelin. (Reproduced from [1] with permission of the American Society for Neurochemistry.)

and neurological abnormalities, both of which become progressively more severe with age [3]. Particularly severe abnormalities occur in the CNS paranodal loops, where glia-axon tight junctions are located. Abnormalities in the PNS of these knockout mice are much less severe. Mice lacking the sulfotransferase that converts cerebroside to sulfatide exhibit similar paranodal disorganization in the CNS, indicating that sulfatide is important for establishing the normal oligodendroglial-axon interactions in the paranodal region [3, 4]. Experiments with cultured oligodendrocytes have demonstrated that both galactocerebroside and sulfatide also have important functions in the differentiation of oligodendrocytes, with sulfatide being particularly important [4].

In addition to cerebroside/sulfatide, the major lipids of myelin are cholesterol and phospholipids [5]. On a molar basis, CNS myelin preparations contain cholesterol, phospholipid, and galactolipid in a ratio varying between 4:3:2 and 4:2:2. Thus, myelin contains substantially more molecules of cholesterol than any other single lipid, although on the basis of weight the content of galactolipids is comparable and total phospholipids are most abundant. The single most prominent phospholipid, which is characteristic of myelin, is ethanolamine-containing plasmalogen (a glycerophospholipid containing an alkenyl ether bond). Cholesterol is enriched on the extracellular face of the myelin membrane, whereas ethanolamine plasmalogen is asymmetrically localized to the cytoplasmic half of the bilayer. CNS myelin contains

some other minor lipids, including polyphosphoinositides, fatty acid esters of galactocerebroside, and two galactosyldiglycerides [5]. Myelin from mammals also contains small amounts of gangliosides (complex sialic acid-containing glycosphingolipids). The major ganglioside in CNS myelin is a monosialoganglioside (GM1), and there are very low amounts of the more complex polysialogangliosides characteristic of neuronal membranes. Myelin from certain species (including human) contains an additional novel ganglioside as a major component: sialosylgalactosylceramide (GM4).

For the most part, peripheral myelin lipids are qualitatively similar to those in CNS myelin, but there are quantitative differences [1]. PNS myelin contains less cerebroside and sulfatide and considerably more sphingomyelin than CNS myelin. LM1 ganglioside, sialosyl-lactoneotetraosylceramide, is a characteristic component of PNS myelin of some species. However, differences in lipid composition are not as dramatic as the differences in protein composition between CNS and PNS myelin described below.

20.2.2 Proteins of CNS Myelin

The protein composition of myelin is simpler than that of most other membranes, with PLP and MBP accounting for about three-fourths of the total in CNS myelin of most species [1, 5]. Numerous other proteins are present in much smaller amounts. The protein composition of rat brain myelin on a sodium dodecyl sulfate (SDS) gel is shown in Figure 20.4. The quantitative predominance of PLP and MBP is clear, although MBP exists in several isoforms of different molecular weights in rodent myelin. These two proteins are major constituents of all mammalian CNS myelin membranes and similar proteins are present in myelin membranes of many lower species.

20.2.2.1 *Proteolipid Protein.* PLP is a very hydrophobic protein with a molecular mass of about 30,000 [6, 7], although it exhibits a lower apparent molecular weight on SDS gels (Fig. 20.4). The amino acid sequence, strongly conserved during evolution, contains four putative membrane-spanning domains. PLP is a tetraspan protein with both the N- and C-termini on the cytoplasmic side of the membrane and two extracellular loops in the intraperiod line, as shown in Figure 20.3. A role for PLP in determining the structure of the intraperiod line of CNS myelin was supported by the finding that this line is abnormally condensed in PLP knockout mice and spontaneously occurring PLP mutants. PLP has an alternatively spliced 20-kDa isoform, DM20, which is present in CNS myelin at lower concentration than PLP (Fig. 20.4) and is identical in sequence, except for a deletion of 35 amino acids. The hydrophobicity of PLP/DM20 is increased by 3 mol of fatty acid per mole of protein in ester linkage at several cysteines.

The PLP gene is expressed very early in development, and in fact DM20 messenger RNA (mRNA) appears earlier than PLP during development, even before myelin formation in embryos and in premyelinating oligodendrocytes [7]. It is thought that it might have a role in oligodendrocyte migration or differentiation in addition to a structural role in myelin. Although PLP and DM20 serve important functions, they are not essential. Contrary to the general expectation that PLP would be needed for formation of compact, multilamellar myelin, a knockout mouse for PLP/DM20 is

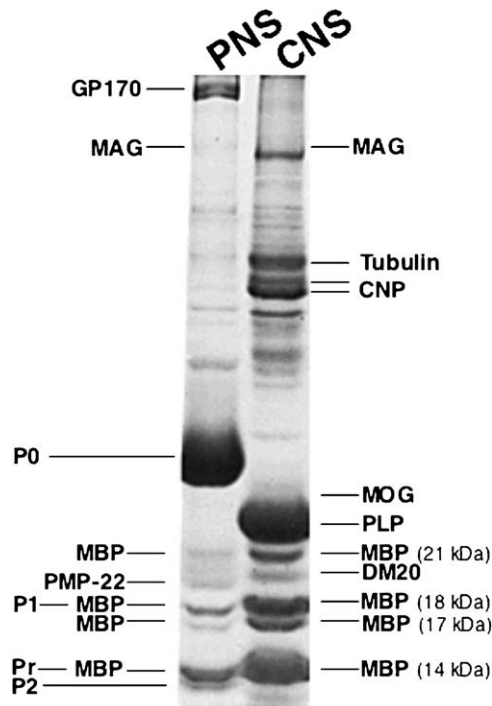


Figure 20.4 Polyacrylamide gel electrophoresis of myelin proteins in presence of SDS. The proteins of isolated rat PNS and CNS myelin are stained with Coomassie brilliant blue. The quantitative prominence of P0 and PLP in PNS and CNS myelin, respectively, is clear. There is a small amount of the smaller PLP isoform, DM-20, in the CNS myelin, and PMP-22 is a relatively minor band in the PNS myelin. The four MBP bands in both lanes are the 14-, 17-, 18-, and 21-kDa isoforms expressed in rats. The 18-kDa MBP and the 14-kDa MBP are also called P1 and Pr, respectively, in the terminology sometimes used for the rodent PNS. The P2 protein, which varies in amount between species and is prominent in bovine and human myelin, is a very minor component of rat PNS myelin migrating just below the 14-kDa MBP (Pr). The 26-kDa MOG is probably the faint band just above PLP in the CNS lane. CNP migrates as a tight doublet, and the lower and upper bands are sometimes referred to as CNP1 and CNP2, respectively. Note that the location shown for MAG (which stains too faintly to be seen well on the gels) is just above a discrete Coomassie blue-stained band in the CNS lane, which is probably the 96-kDa subunit of Na^+ , K^+ -ATPase. GP170 is a 170-kDa glycoprotein present in PNS myelin. Tubulin is a relatively prominent component in isolated CNS myelin and is probably associated with oligodendroglial membranes in the preparations. (Reproduced from lanes C and D of Fig. 4-12 in [1] with permission of the American Society for Neurochemistry.)

initially relatively normal with respect to myelin formation (except for the difference in the intraperiod line spacing), life span, and motor performance [7]. This suggests that other proteins or lipids of myelin may contribute to adherence of the extracellular faces of the bilayers at the intraperiod line. On the other hand, myelin in the PLP-null mutant is extra sensitive to osmotic shock during fixation, suggesting that PLP does enhance the stability of myelin, possibly by forming a “zipperlike” structure after it is compacted. Furthermore, in older PLP/DM20 knockout mice, there is significant axonal degeneration, suggesting that while myelin can form in the

absence of PLP/DM20, CNS myelin devoid of PLP/DM20 cannot sustain normal axonal function. Despite the apparent similarity of the PLP and DM20, DM20 cannot replace PLP in transgenic mice [8]; that is, the same long-term axonal degeneration occurs in mice expressing exclusively DM20 protein. Thus, PLP has selective and apparently important functions in the CNS relative to DM20. While the loss of PLP/DM20 has clear neuropathological consequences in older animals, the loss of these proteins is significantly less serious than expression of mutated or excess PLP/DM20. Both human patients [9] and genetically engineered or naturally occurring animal mutants [10] with defects in the PLP gene exhibit hypomyelination and often early death. This may result from production of either abnormal protein that cannot fold correctly or simply increased amounts of normal PLP [7], which induce an unfolded protein response and are toxic to oligodendrocytes. While PLP/DM20 expression is highest in oligodendrocytes in the CNS, PLP/DM20 is also expressed in myelinating Schwann cells, although not incorporated into PNS myelin in appreciable amounts. It is also expressed in nonmyelinating Schwann cells, and low levels of DM20 expression have been found in thymus and heart [11], again suggesting that this protein has other functions unrelated to formation and maintenance of myelinated axons.

20.2.2.2 Myelin Basic Protein. MBP is a positively charged extrinsic membrane protein which is soluble in aqueous solutions and can be extracted from myelin with either dilute acid or salt solutions. The MBP genes from a number of species are highly conserved and are alternatively spliced producing a substantial number of isoforms [11, 12]. In rodent myelin, 14- and 18-kDa isoforms are most abundant (Fig. 20.4), whereas the 18-kDa isoform is most prominent in humans. MBPs are localized at the cytoplasmic surface in the major dense line of myelin (Fig. 20.3), a conclusion based on inaccessibility to surface probes and direct localization at the electron microscope level by immunocytochemistry. There is evidence to suggest that MBP forms dimers, and it is believed to be the principal protein stabilizing the major dense line of CNS myelin, possibly by interacting with negatively charged lipids. A severe hypomyelination and failure of compaction of the major dense line in MBP-deficient *shiverer* mutants supports this hypothesis [11]. The MBP isoforms exhibit additional microheterogeneity upon electrophoresis in alkaline conditions. This is due to a combination of phosphorylation, loss of the C-terminal arginine, and deamidation. There is also heterogeneity in the degree of methylation of an arginine at residue 106. The rapid turnover of the phosphate groups present on many of the MBP molecules [13] suggests this posttranslational modification might influence the close apposition of the cytoplasmic faces of the membrane (whether phosphorylation modifies this process in a dynamic manner is a topic of speculation). The physiological significance of the heterogeneity of MBPs, which results from alternative splicing and from unique posttranslational modifications, remains to be established.

Intriguingly, the classical MBP gene is actually part of a larger gene, *golli* (gene of the oligodendrocyte lineage), which is greater than 100 kb in length [12]. This gene has three transcription start sites, two of which are used to transcribe the MBP mRNAs, while the most 5' transcription start site generates golli mRNAs. Transcripts from this upstream promoter are expressed more ubiquitously than MBP mRNAs. Thus, they are expressed in neurons and oligodendrocytes in the nervous system and in T cells in the immune system. Most interestingly from an evolutionary

perspective, the golli proteins contain a 133-amino-acid domain that contains both unique golli sequences and classic MBP sequences. The golli proteins are expressed during embryonic development and in postnatal tissue, and the proteins are found in multiple subcellular localizations, including nuclei, cytoplasm, and cellular processes. Their function is not yet understood, although there is the suggestion that they may be involved in process extension in neural cells [11, 12].

It should also be noted that there is another protein in CNS myelin with a similar name but no sequence homology to the major MBP. It is the myelin-associated oligodendrocytic basic protein, which is localized in the major dense line in several 8- to 12-kDa isoforms and appears to function in controlling axonal diameter and the arrangement of the radial component [14].

20.2.3 Proteins of PNS Myelin

The principal difference in protein composition between PNS and CNS myelin is that the major integral membrane protein of PNS myelin is P0, while there is very little or no PLP in PNS myelin (Fig. 20.4) [1, 5]. P0 accounts for over half the total protein in peripheral myelin. Other prominent proteins in compact PNS myelin include PMP-22, MBP, and P2. The 18- and 14-kDa MBP isoforms in PNS myelin are sometimes referred to as P1 and Pr, respectively. The P0, P1 (Pr), and P2 terminology originally came from the relative positions of prominent PNS myelin proteins on SDS gels [15].

20.2.3.1 Protein Zero. P0 is a 30-kDa type I transmembrane glycoprotein with a single extracellular immunoglobulin (Ig)-like domain and one site for N-linked glycosylation (Figs. 20.3 and 20.5) [1, 5, 16, 17]. The glycans at the single glycosylation site of P0 are very heterogeneous. Many are sulfated and include oligosaccharides terminating in sialic acid or sulfated glucuronic acid. The sulfated glucuronic acid on P0 is a key part of the HNK-1 carbohydrate epitope, which is shared with human natural killer cells, and has been implicated in cell-cell adhesion. In addition, there are developmental changes in the composition of the oligosaccharides on P0 [18] that could be important for its function in myelination.

P0 stabilizes the intraperiod line of compact PNS myelin by homophilic interactions (Fig. 20.3) [1, 17]. The first evidence for this was that expression of P0 in transfected cells caused cell-cell interactions which were due to homophilic binding of its extracellular domains. The relatively large, glycosylated, extracellular Ig-like domain of P0 probably accounts for the greater separation of extracellular surfaces in PNS myelin relative to CNS myelin where closer apposition of these surfaces is possible in the presence of the smaller extracellular domains of PLP. Evidence reviewed in [17] suggests that homophilic interactions between P0 molecules involve both protein-protein and protein-carbohydrate interactions. Furthermore, investigation of the crystal structure of the P0 extracellular domain suggests that P0 molecules cluster on each membrane surface as tetramers. The crystal structure also suggested that a tryptophan residue at the apex of the extracellular domain could interact directly with the lipid bilayer of the apposing membrane. P0 also has a relatively large positively charged domain on the cytoplasmic side of the membrane that contributes significantly to stabilization of the major dense line in the PNS. Part of the evidence for this difference is that, although the major dense line does not

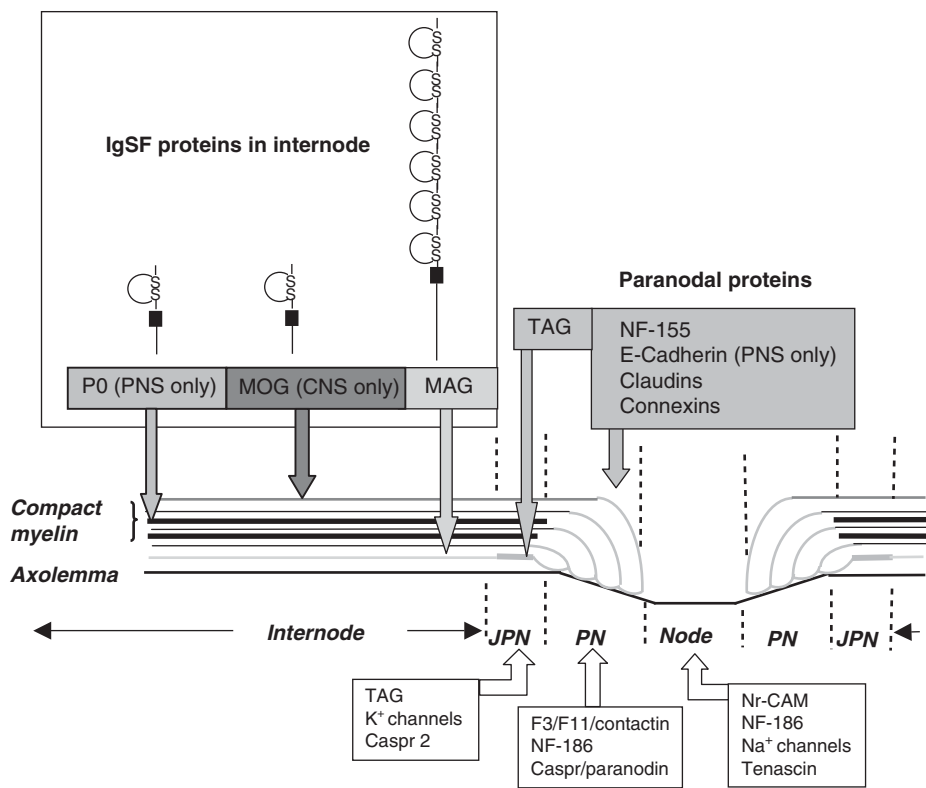


Figure 20.5 Locations of proteins in glial and axonal membrane domains of myelinated axons. Colored boxes and arrows designate the specific localizations of proteins in the various domains of the glial membranes, which are drawn with the same color. The nodal regions, which separate the segments of myelin (internodes) on myelinated axons, are divided into distinct domains with differing structures and biochemical compositions; the node itself where ion fluxes generating the action potential occur, paranodes (PNs) with glial lateral loops that make tight junctions with the axonal surface membrane (axolemma), and juxtaparanodes (JPNs). The internodes include the compact myelin as well as the glial membranes at the outside (abaxonal) and inside (adaxonal) of the myelin sheath. Many of the proteins selectively localized in these domains are members of the immunoglobulin superfamily (IgSF) and participate in glia–axon interactions. The structures of IgSF proteins in the myelin internode are shown in the box at the upper left. The circular structures in these proteins represent Ig-like domains, and the black rectangles represent transmembrane domains. These proteins are selectively localized at sites shown by the arrows: P0, compact myelin (gray); MOG, abaxonal membrane (red); and MAG, adaxonal membrane (yellow). Proteins specifically expressed in axonal membranes of the JPN, PN, and node are listed in the boxes at the bottom. IgSF members contribute to the formation and maintenance of these structures by interacting with other membrane or cytoskeletal proteins. Abbreviations: caspr, contactin-associated protein; Nr-CAM, neuron–glia-related cell adhesion molecule; NF, neurofascin; TAG, transiently expressed axonal surface glycoprotein. [Adapted with permission from R.H. Quarles (2003). In *Encyclopedia of Life Sciences*, M. Cox, I. Levitan, P. Delves, Eds., Wiley, New York, Figure 3, (DOI:10.1038/ngp.els.0003814).] (See color insert.)

compact normally in CNS myelin of mutant Shiverer mice that lack MBP, the structure of PNS myelin is relatively normal in these mutants.

The complete knockout of P0 has profound consequences for myelin structure, in contradistinction to the previously noted, relatively benign CNS consequence of deletion of the PLP gene. P0-null mice exhibit abnormal motor coordination, tremors, occasional convulsions, and a severe hypomyelination with thin noncompacted myelin sheaths. Furthermore, expression of the correct amount of P0 is also essential for normal myelin formation and maintenance. Young mice heterozygous for the P0-null mutation appear normal but develop progressive demyelination with age, which resembles chronic inflammatory demyelinating neuropathy and may involve autoimmune mechanisms [19, 20]. Furthermore, transgenic mice overexpressing P0 exhibit a dose-dependent dysmyelinating neuropathy ranging from a transient hypomyelination to a severe arrest of myelination and impaired sorting of axons [21]. The critical dosage of P0 required for normal myelin formation is similar to observations with other myelin proteins and may reflect the necessity for appropriate amounts of myelin proteins to form stoichiometric complexes in compact myelin. However, in the case of P0, the pathology that occurs with overexpression may also reflect a mistargeting of the protein and an interesting misuse of its obligate homophilic adhesive properties. Some of the extra P0 is inappropriately located in normally dynamic mesaxonal membranes, causing them to adhere like compact myelin and halting myelination [22]. Furthermore, there are a growing number of reports of inherited human neuropathies affecting myelin in which the P0 gene is mutated [20, 23, 24]. These include severely affected Dejerine–Sottas patients and the less severely affected Charcot–Marie–Tooth (CMT) type 1B patients. Research on animal models in which the expression of P0 has been genetically modified provide important clues about the pathogenesis of these human disorders [24, 25].

It is well established that expression of most of the P0 in peripheral nerve during development parallels the accumulation of myelin, which is consistent with its important structural role in compact myelin. However, it should be noted that low basal levels of P0 are expressed in Schwann cells and neural crest cells early in embryonic development well before myelination, suggesting that P0 could be an early marker of the glial lineage and perform other functions that are unrelated to myelination. Because this review focuses on myelin, this early expression and other potential functions for P0 will not be covered comprehensively here but are reviewed and discussed in more detail elsewhere [26, 27]. The other potential functions include Schwann cell–axon interactions and signal transduction. The cytoplasmic domain of P0 is phosphorylated on serine and tyrosine residues [13, 28], and this might be indicative of signaling mechanisms within Schwann cells during early development as well as later during myelination.

20.2.3.2 *Peripheral Myelin Protein-22.* Similarly to P0, PMP-22 is a glycoprotein of compact PNS myelin with a single site for N-linked glycosylation [16, 29]. However, it has a very different overall structure from P0 and accounts for less than 5% of total PNS myelin protein. This 22-kDa glycoprotein is a tetraspan protein (Fig. 20.3) like the major PLP of CNS myelin, but there is no sequence homology to PLP. Also, unlike P0, which is nerve specific, PMP-22 is expressed in other tissues, including lung, gut, and heart. PMP-22 was cloned as a gene whose expression is downregulated,

similarly to other myelin proteins, in association with Schwann cell proliferation following nerve transection. PMP-22 mRNA codes for the same protein as a growth arrest-specific mRNA (gas-3) that had been cloned previously from fibroblasts. It is in a highly homologous family of small hydrophobic tetraspan proteins that also include epithelial membrane proteins (EMP-1, -2 and -3) [30]. The oligosaccharide moieties on PMP-22 are of the complex type [31] and in some species include the adhesion-related HNK-1 carbohydrate epitope.

PMP-22 has received much attention since its cloning because abnormalities of its gene cause the dysmyelinating phenotypes in *trembler* mice and several neuropathies in humans (see below). Therefore, it is clear that this protein must play a crucial role in the formation or maintenance of myelin. The *trembler* and less severe *trembler-J* murine phenotypes are caused by two different dominant point mutations in PMP-22 and are characterized by hypomyelination, continued Schwann cell proliferation, and partial paralysis. Although the tetraspan PMP-22 is localized primarily in compact PNS myelin as shown in Figure 20.3, it is not known if its extracellular or cytoplasmic domains play an important structural role for myelin. Because it is a quantitatively relatively minor component of compact myelin, it may have a more dynamic function. Nevertheless, its primary localization within compact myelin suggests that an important aspect of its function occurs at this location. Furthermore, PMP-22 has been shown to form complexes with P0 in myelin membranes, and this interaction with P0 may be relevant to its function [32, 33]. The association of PMP-22 with growth arrest in Schwann cells and other cell types suggests that it may have an unknown role in regulation of growth or differentiation of Schwann cells. The human neuropathies caused by abnormalities of the PMP-22 gene include CMT disease type 1A, which is usually caused by duplication of the gene and sometimes by point mutations. CMT1A usually has an onset in the second or third decade of life and is characterized by segmental demyelination and remyelination. A milder hereditary neuropathy with liability to pressure palsies is brought on by pressure or trauma to an affected nerve and is caused by heterozygous deletion of the PMP-22 gene. These human neuropathies caused by over- and underexpression of PMP-22 [33, 34], respectively, point to the fact that correct dosage of this protein is necessary for normal formation and maintenance of myelin. The importance of dosage is also clearly shown by a variety of genetically engineered mice expressing different amounts of PMP-22 and serving as useful models for the human neuropathies [34]. Studies on murine mutants and the human neuropathies suggest that an important aspect of the pathology may involve abnormal accumulation of PMP-22 in the Schwann cell endoplasmic reticulum rather than its being transported to the myelin membrane [33, 34].

20.2.3.3 Myelin Basic Protein. In the PNS, MBP only accounts for 5–18% of total myelin protein in various species, in contrast to the CNS, where it is close to one-third of the total [5]. In adult rodents, the 14-kDa MBP is the most prominent MBP isoform and is sometimes referred to as Pr in the PNS nomenclature for rodents (Fig. 20.4). The 18-kDa component is also present and is sometimes referred to as P1. It appears that MBP does not play as critical a role in myelin structure in the PNS as it does in the CNS. For example, in contrast to the severe hypomyelination and failure of compaction of the major dense line in CNS myelin of the MBP-deficient Shiverer mutant (see above), the PNS myelin in this mutant is essentially normal, in

both amount and structure. This CNS/PNS difference in the role of MBP is probably because the cytoplasmic domain of P0 has an important role in stabilizing the major dense line. However, animals doubly deficient for P0 and MBP have a more severe defect in compaction of the PNS major dense line than P0-null mice, which indicates that both proteins contribute to compaction of the cytoplasmic surfaces in PNS myelin [17].

20.2.3.4 Protein 2. PNS myelin contains a 15-kD positively charged protein different from MBP that is referred to as P2. It is unrelated in sequence to MBP and is a member of a family of cytoplasmic fatty acid binding proteins (FABPs) that are present in a variety of cell types [35]. The amount of P2 is variable among species, accounting for about 15% of total protein in bovine PNS myelin, 5% in humans, and less than 1% in rodents. P2 is generally considered a PNS myelin protein, but it is expressed in small amounts in CNS myelin sheaths of some species. P2 appears to be present in the major dense line of myelin sheaths where it may play a structural role similar to MBP (Fig. 20.3). Interestingly, the larger amounts of P2 that are in myelin of some species correlate with increased widths of the major dense lines as determined by X-ray diffraction, and there appears to be substantially more P2 in large sheaths than small ones [2]. The large variation in the amount and distribution of the protein from species to species and sheath to sheath raises so far unanswered questions about its function. Its similarities to cytoplasmic proteins in other cells, whose functions appear to involve solubilization and transport of fatty acids and retinoids, suggest that it might function similarly in myelin assembly or turnover, but there is little experimental evidence to support this hypothesis.

20.2.4 Other Proteins in Myelin and Myelin-Related Membranes

The principal structural proteins of compact myelin are described above. However, there are numerous other quantitatively less prominent proteins that are associated with myelin sheaths (see Fig. 20.4). Some of these are in the compact myelin itself, but many are selectively localized in other glial membranes within the sheath, where they have important functions for the formation and maintenance of myelinated axons. Some of these are discussed in this section.

20.2.4.1 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase. A characteristic component of purified CNS myelin is the enzyme CNP [36], which runs as a 46- and 48-kDa doublet on SDS gels (Fig. 20.4). Although there are low levels of CNP associated with other cell types, it is greatly enriched in CNS myelin and oligodendrocytes, for which it is a commonly used biochemical marker. It is expressed at a much lower concentration in Schwann cells at the onset of myelination and does not increase during development with the accumulation of myelin as in the CNS. The enzyme is extremely active with the substrate 2',3'-cyclic adenosine monophosphate (cAMP) as well as cyclic guanosine monophosphate (cGMP), cyclic cytosine monophosphate (cCMP) and cyclic uridine monophosphate (cUMP) analogs, which are all hydrolyzed to the corresponding 2'-isomer. This is likely to be a nonphysiological activity, because it is the 3',5' cyclic nucleotides that have been shown to have biological activity. Nevertheless, evolutionary conservation of the catalytic site indicates that its amino acid sequence likely has an important function, although the precise role of

CNP has remained elusive over the many years since it was discovered. Two CNP polypeptides are generated by alternative splicing of the mRNA, with the larger polypeptide having an extra 20 amino acids at the N-terminus. CNP is not a major component of compact myelin but is concentrated in specific regions of the myelin sheaths associated with cytoplasm, such as the oligodendroglial processes, inner and outer tongue processes, and lateral loops. The protein is in the cytoplasm, but much of it associates with membranes, because both isoforms are isoprenylated at the C-terminus and acylated. Some clues about its function have come from reports that it binds to cytoskeletal elements such as F-actin and tubulin and that overexpression in cultured nonneural cells promotes outgrowth of processes. Such findings suggest that its function may be in regulating cytoskeletal dynamics to promote process outgrowth and differentiation in oligodendrocytes. Furthermore, aberrant myelination occurring in vivo in transgenic mice overexpressing CNP similarly suggests that it could be an early regulator of cellular events that culminate in CNS myelination. However, it is also important to note that the amino acid sequence of CNP puts it in a superfamily of RNA-processing enzymes whose physiological roles are unclear, so the relevance of this to oligodendrocytes and myelination is also unclear. An interesting possibility combining some of the above information is that CNP could be involved in some specialized aspects of RNA transport and/or processing in oligodendrocytes. Yet most puzzling of all is the phenotype displayed by the recently generated CNP-null mice, which appear to myelinate entirely normally but as adults exhibit axonal swelling, neurodegeneration, and premature death. It has been speculated that CNP is a multifunctional protein with an initial role in oligodendroglial differentiation that can be compensated for by another protein and a second function essential for the normal interaction of oligodendrocytes with axons leading to axonal degeneration in its absence [36]. Clearly, more research is needed to fully understand the functions of this intriguing myelin/oligodendrocyte-related protein.

20.2.4.2 Glycoproteins Present in Small Amounts in Internodes of Myelin Sheaths

20.2.4.2.1 Myelin-Associated Glycoprotein. MAG is a quantitatively minor, 100-kDa glycoprotein in purified CNS and PNS myelin [16, 37] that electrophoreses at the position shown in Figure 20.4. However, because of its small amount (<1% of total protein) and weak staining by Coomassie blue, it does not correspond to one of the discrete protein bands visible in the figure. MAG has a single transmembrane domain that separates a heavily glycosylated extracellular part of the molecule, composed of five Ig-like domains and eight or nine sites for N-linked glycosylation, from an intracellular, carboxy-terminal domain (Fig. 20.5). MAG in rodents occurs in two developmentally regulated isoforms, which differ in their cytoplasmic domains and are generated by alternative splicing of its mRNA. The isoform with a longer C-terminal tail (L-MAG) predominates early in development during active myelination of the CNS, whereas the isoform with a shorter cytoplasmic tail (S-MAG) increases during development to become prominent in adult rodents. S-MAG is most prominent in the PNS at all ages.

MAG is not present in compact, multilamellar myelin but is located in the periaxonal glial membranes of myelin sheaths (Figs. 20.2 and 20.5). This location next to the axon and its membership in the Ig superfamily suggest that it functions in adhesion and signaling between myelin-forming cells and the axolemma. Indeed, substantial evidence has now accumulated that MAG is involved in signaling in both

directions between glia and axons, although its most important functions appear to be different in the CNS and the PNS. MAG is in the “siglec” (sialic acid binding immunoglobulin-like lectins) subgroup of the Ig superfamily and binds to glycoproteins and gangliosides with terminal $\alpha 2,3$ -linked sialic acid moieties. Thus, some of the axolemmal binding partner(s) for MAG are likely to be sialoglycoconjugates. A relationship of MAG to other adhesion proteins also is demonstrated by the presence in most species of a sulfate-containing epitope in its oligosaccharide moieties that reacts with the HNK-1 monoclonal antibody. The carbohydrate HNK-1 epitope is expressed on many neural adhesion proteins, including N-CAM and MAG, and has been shown to function in cell–cell interactions.

MAG is not essential for myelin formation because MAG-null mice myelinate relatively normally. Nevertheless, in the CNS, these knockouts exhibit a significant delay of myelination, periaxonal and paranodal structural abnormalities, redundant myelin loops, and supernumerary myelin sheaths. In addition, there is degeneration of periaxonal oligodendroglial processes in aging MAG-null mice, suggesting the occurrence of a “dying-back oligodendroglialopathy.” Therefore, the absence of MAG causes oligodendrocytes to form myelin less efficiently during development and become dystrophic with aging. Furthermore, although the neurological deficit in MAG-null mice is mild, double knockouts in which the absence of MAG is combined with the genetic ablation of other proteins results in more severe CNS phenotypes than either knockout alone. These *in vivo* findings suggest that MAG-mediated signaling from axons to oligodendrocytes is needed for efficient myelination and maintenance of healthy mature oligodendroglia. As with other proteins in the Ig superfamily, it is likely that the interaction of MAG with its ligand(s) on the axolemma mediates cell–cell signaling by mechanisms involving phosphorylation. The cytoplasmic domains of MAG are phosphorylated on serine and threonine residues by protein kinase C, and L-MAG is also phosphorylated on tyrosine-620. Furthermore, the cytoplasmic domain of L-MAG has been shown to interact with Fyn tyrosine kinase, phospholipase C γ , and other oligodendroglial proteins. A role for MAG signaling within oligodendrocytes was supported by triggering specific signaling events involving phosphorylation when MAG was crosslinked on the cell surface with antibodies [38]. The L-MAG isoform appears to be particularly important for CNS myelination, because genetically engineered mice lacking only the L isoform exhibit the same CNS abnormalities as total knockouts but not the PNS pathology of total knockouts described below.

In the PNS, MAG is present in the Schwann cell membranes of the Schmidt–Lanterman incisures, paranodal loops, and the outer mesaxon in addition to the periaxonal Schwann cell membranes [16, 37]. Therefore, in addition to a role in Schwann cell–axon interactions, MAG may also function in interactions between adjacent Schwann cell membranes at these other locations in the PNS. PNS myelination in MAG-null mice is initially more normal than CNS myelination. However, as the mice age, they develop a peripheral neuropathy characterized by degeneration of myelinated axons, which is the most severe phenotypic abnormality displayed by the knockout mice. The pathology is associated with decreased axonal caliber, increased neurofilament density, reduced expression and phosphorylation of neurofilaments, and eventually axonal degeneration. These findings demonstrate an essential role for MAG in signaling from Schwann cells to axons that is necessary for the maintenance of normal myelinated axons in the PNS. Thus, MAG is another

example of a myelin-related, glial protein whose absence has profound effects on the ensheathed axon.

In this regard, it is of interest that MAG is one of several white matter proteins (also including Nogo and the oligodendrocyte-myelin glycoprotein) that inhibit neurite outgrowth in tissue culture and axonal regeneration in vivo. This inhibitory activity has been studied intensively in recent years, since it is important for understanding factors that prevent axonal regeneration following neural injury [39]. This area of research has led to remarkable progress in identifying neuronal MAG receptors and to the identification of a MAG-mediated signaling mechanism that affects neurons and also could be important for the normal maintenance of myelinated axons. Thus, a physiologically important signal promoting the stability of mature myelinated axons could be interpreted inappropriately by a plastic developing neurite in vitro or a regenerating neurite in vivo, thereby inhibiting its growth. The MAG receptor on neurites that transmits this inhibitory signal appears to be a complex localized in raft-like signaling domains which consists of gangliosides, the glycosylphosphatidylinositol-anchored Nogo receptor, and the p75 neurotrophin receptor. However, it should be noted that recent studies on mice in which the Nogo receptor was genetically deleted [40] suggest that the signaling pathways whereby MAG and other myelin constituents inhibit neurite outgrowth may be more complex than currently understood. Nevertheless, the neuronal receptor complex involved in MAG's effects on neurite outgrowth is also likely to function within myelinated axons to promote axonal stability, but this remains to be established.

It is noteworthy that the axonal degeneration that occurs in the PNS of MAG-null mice is not observed in the CNS, possibly because other CNS myelin proteins enhance axonal stability. These could include PLP and/or CNP, both of which are needed for axonal stability in the CNS where they are present in much higher concentration. In summary, it appears that the most important function of MAG in the PNS is transmitting a signal from Schwann cells to axons that is needed for the stability of myelinated axons, whereas its principal function in the CNS is to transmit a signal in the reverse direction that promotes efficient myelination and oligodendrocyte vitality.

20.2.4.2.2 Myelin Oligodendrocyte Glycoprotein. MOG is a 26- to 28-kDa glycoprotein that was originally detected in brain by a monoclonal antibody raised to rat cerebellar glycoproteins [16, 41]. Similarly to MAG, it is a member of the Ig superfamily that is a quantitatively minor component of myelin sheaths that is localized in membranes distinct from compact myelin. However, unlike MAG it is not expressed in the PNS, and its localization in the CNS is very different from periaxonal MAG. It is expressed preferentially on the outside surface of myelin sheaths and oligodendrocytes (Figs. 20.2 and 20.5), where it is well situated to interact with components in the extracellular environment. It has a single Ig-like variable region domain, one site for N-linked glycosylation, and two hydrophobic, potential transmembrane domains (Fig. 20.5). However, topographical studies have indicated that the second hydrophobic domain does not transverse the membrane completely, so the carboxy terminus of MOG is in the oligodendroglial cytoplasm. It is of interest that the cytoplasmic domain of MOG contains targeting signals that direct it to the basolateral domain of Madine-Darby canine kidney (MDCK) cells and presumably account for its selective localization on the surface of oligoden-

drocytes and myelin sheaths. The oligosaccharide moieties on MOG are primarily of the complex type, and a subset of the molecules contains the adhesion-related HNK-1/L2 epitope.

The function of MOG is not known, although its localization on the surface of oligodendrocytes and myelin suggests that it could function in the transmission of signals from the extracellular environment to oligodendrocytes. Such a function is supported by changes in cultured oligodendrocytes when MOG is crosslinked on the cell surface with anti-MOG antibodies [42, 43]. The developmental pattern of MOG is delayed somewhat in comparison to other myelin proteins, indicating that its primary functional role may relate to mature oligodendrocytes. In a review [42], three possible functions for MOG were proposed. The first was that it could be an adhesion protein, perhaps mediating an interaction between the surfaces of neighboring mature myelin sheaths, which does not occur in the PNS. The second was a role in modulating the stability of the oligodendroglial microtubules. They summarized experimental results suggesting that the interaction of MOG with an unknown extracellular ligand could regulate microtubular stability with MBP acting as an intermediary molecule. The third suggested function was that MOG binding to the C1q component of complement is responsible for the capacity of CNS myelin to activate complement. Although the role of this phenomenon in normal physiology is not obvious, it could be related to inflammatory processes occurring in demyelinating diseases such as multiple sclerosis. However, its physiological function remains obscure, because the recent generation of MOG-null mice yielded an apparently normal phenotype [44]. On the other hand, much of the recent research on MOG has built a strong case that it may play a key role in the autoimmune aspects of MS, as discussed below.

20.2.4.2.3 Oligodendrocyte Myelin Glycoprotein. Another glycoprotein related to myelin with a similar name to MOG is the oligodendrocyte myelin glycoprotein (OMgp) [45]. It was first characterized as a 120-kDa phosphatidylinositol-linked glycoprotein in human white matter and subsequently cloned. It is not a member of the Ig superfamily but is characterized by a cysteine-rich motif at the N-terminus, a series of tandem leucine-rich repeats, and the HNK-1 epitope. These properties suggest that it may function in cell–cell interactions. However, unlike MAG and MOG, it is not specific to myelin-forming cells and is also expressed in neurons. It has attracted substantial interest in recent years because it is one of the myelin-associated inhibitors of axonal regeneration [39] (see above), but its function with regard to myelination is unclear at this time.

20.2.4.3 Tetraspan Proteins. Some tetraspan proteins present in substantial amounts in compact myelin are PLP/DM20 and PMP-22, which have already been discussed. Interestingly, there are numerous other tetraspan proteins in myelin and related glial membranes [46], including myelin and lymphocyte protein (MAL/MVP17/VIP17) and plasmalipin in compact myelin, as well as claudins, CD9, and connexins in myelin-related glial membranes. The presence or absence of these proteins can be essential to the specialized structure and function of myelin sheaths. The paranodal loops, which form the tight junctions between glial processes and axons in the paranodal regions of the sheaths (see Figs. 20.1 and 20.5), are crucial for normal firing of myelinated axons. Rapid saltatory conduction of nerve impulses in myelinated fibers is, thus, dependent

on the structural integrity of nodes of Ranvier and of the tight junctions at this location, which prevent ion leaking into the internodes.

20.2.4.3.1 Myelin and Lymphocyte Protein and Plasmolipin. The 17-kDa MAL protein was first identified in compact myelin as MVP17, a novel myelin membrane protein [47], which was soon demonstrated to be identical to MAL and VIP17. MAL (MVP17/VIP17) is part of the apical sorting machinery in nonneural polarized cells, and it has been suggested that it is involved in protein sorting in myelin membrane domains. It associates with glycosphingolipid-enriched protein/lipid rafts and may function in their sorting and transport to myelin [48, 49]. MAL is part of an extended gene family, which includes plasmolipin, another myelin tetraspan protein [50]. Plasmolipin is also associated with glycosphingolipid-enriched membrane domains from myelin. Therefore, these two proteins may be involved in sorting of proteins or signal transduction through lipid rafts in myelin.

20.2.4.3.2 Claudin Family. Claudins are a family of tight junction proteins found in many tissues which form barriers to the diffusion of solutes between adjacent cells. Tight junctions in the paranodal regions of the PNS (Figs. 20.1 and 20.5) that act as barriers for diffusion of small ions involve the tetraspan claudin-5 [51]. The oligodendrocyte specific protein (OSP) is found in the radial component of myelin; it was initially identified by differential screening as a novel tetraspan protein found in oligodendrocytes, but it was quickly established also to be a member of the claudin family, that is, claudin-11 [52]. The radial component is a specialized ultrastructural feature in CNS myelin, but not PNS myelin, which appears as lines of tight junctions with reduced spacing between extracellular leaflets. These lines of tight junctions extend in spiraled fashion across the whole thickness of CNS myelin sheaths from one paranodal region to the other. The CNS myelin tight junctions between adjacent layers of spiraled membranes probably contribute stability and, most importantly, act as a barrier to the diffusion of ions that is essential for the normal electrophysiological function of myelinated axons. The principal protein component of these tight junctions is OSP/claudin-11 [52], and in OSP/claudin-11-null mice, these tight junctions are missing from CNS myelin. Thus, OSP/claudin-11 is essential for formation of the radial component in CNS myelin. In addition to its role in the formation of tight junctions, OSP/claudin-11 is also involved in oligodendrocyte migration, possibly through its interactions with OSP/claudin-11-associated protein (OAP)-1 and β_1 integrin [52].

20.2.4.3.3 CD9. CD9 is a well-characterized hematopoietic tetraspan protein that also has been shown to be present in CNS and PNS myelin, although it is present at higher levels in PNS myelin. In other cells, it is involved in integrin signaling, cell adhesion, and motility. It is expressed at late stages of myelination and in the CNS is primarily found in paranodal junctions [53]. While compact CNS myelin is apparently normal in CD9-null animals, the paranodal loops are often disconnected from axonal membranes, and the transverse bands of the paranodal loops are lost. In the PNS, in addition to altered paranodes, hypermyelination occurs. Thus, this tetraspan protein is crucial for the formation and maintenance of normal paranodal junctions.

20.2.4.3.4 Connexins. Gap junctions are another type of membrane contact in the paranodal regions which provide a radial pathway for diffusion of small molecules across the lateral loops. In particular the tetraspan proteins connexin-32 and connexin-29 are found in myelin [54], predominantly in noncompact domains of myelin, including paranodes and Schmidt–Lanterman incisures (Figs. 20.1 and 20.5). While gap junctions typically form between adjacent cells, in myelin they form between adjacent layers of membrane. In Schwann cells, functional gap junctions provide a radial pathway of interconnection throughout the myelin. It has been proposed that this radial pathway through the myelin mediates spatial buffering of extracellular potassium during action potential activity as well as communication from the adaxonal domain of myelin to the cell body. Similar radial pathways containing gap junctions likely also exist in CNS myelin. Mutations of connexin-32 are associated with the peripheral neuropathy CMT disease type X but interestingly cause little CNS pathology.

20.2.4.4 Other Paranodal Proteins. Several of the tetraspan proteins discussed in the previous section are selectively localized in the paranodal membranes of myelin sheaths, and there are a large number of other proteins that also have important functions at this location. The overall structure of the axonal membrane at the nodes of Ranvier themselves is essential for normal axonal firing, and this appears to be regulated in trans by the proteins and lipids in the paranodal myelin membranes. The axonal and glial membranes in this region of the fiber demonstrate an exquisite division into highly specialized domains (Fig. 20.5), whose biochemical structures are currently a very active subject of research. The correct positioning of sodium channels exclusively at the nodes and not in the internodes is necessary for generating action potentials. Similarly, the potassium channels are localized very specifically to the juxtaparanodal axonal membrane, and as already noted, cerebroside- and sulfatide-null mice have disorganized paranodes and consequently altered nodal membranes, for example, altered sodium channel organization. A variety of membrane proteins, including several members of the immunoglobulin superfamily, are selectively localized in these nodal and paranodal domains and must play important roles in the formation and stabilization of these complex structures. For example, an important *trans* interaction at the paranode occurs between neurofascin-155 on the glial membrane and contactin/Caspr (contactin-associated protein) multimers on the axonal membrane to form septatelike junctions. A detailed description of the proteins and lipids in these structures is beyond the scope of this chapter, and the reader is referred to excellent recent reviews of this area that are available elsewhere [51, 55].

20.2.4.5 Enzymes Associated with Myelin. A large number of enzymes have been detected in purified myelin [56]. Some appear to be in compact myelin itself, but others may be primarily in associated glial membranes. A few enzymes, such as CNP, are fairly specific for myelin/oligodendrocytes. In addition, a unique pH 7.2 cholesterol ester hydrolase is enriched in myelin. On the other hand, there are many enzymes that are not myelin specific but appear to be authentic components of myelin and not in contaminants. These include cAMP-stimulated kinase, calcium/calmodulin-dependent kinase, protein kinase C, a neutral protease activity, and phosphoprotein phosphatases. The protein kinase C and phosphatase activities are

presumed to be responsible for the rapid turnover of MBP phosphate groups. A PLP acylation enzyme activity is also intrinsic to myelin. Myelin enzymes involved in lipid metabolism include a number of steroid-modifying enzymes and cholesterol esterifying enzymes, UDP-galactose:ceramide galactosyltransferase, and enzymes of glycerophospholipid metabolism, including all those necessary for phosphatidyl ethanolamine synthesis from diradyl-*sn*-glycerol and ethanolamine. The extent of the contribution of these enzymes in myelin (relative to enzymes within the oligodendroglial perikaryon) to metabolism of myelin lipids is unclear. Nevertheless, the findings imply that myelin is metabolically active in the synthesis, processing, and metabolic turnover of some of its own components.

Other enzymes present in myelin include those involved in phosphoinositide metabolism: phosphatidylinositol kinase, diphosphoinositide kinase, the corresponding phosphatases, and diglyceride kinases. These are of interest because of the high concentration of polyphosphoinositides of myelin and the rapid turnover of their phosphate groups. This area of research has expanded toward characterization of signal transduction system(s), with evidence of G proteins, and phospholipases C and D in myelin. Carbonic anhydrase has generally been considered a soluble enzyme and a glial marker, but myelin accounts for a large part of the membrane-bound form in brain. This enzyme may play a role in removal of carbonic acid from metabolically active axons. The enzymes 5'-nucleotidase and Na^+ , K^+ -adenosine triphosphatase (ATPase) have long been considered specific markers for plasma membranes and are found in myelin at low levels. Na^+ , K^+ -ATPase is involved in transport of monovalent cations, and its presence suggests that myelin may have an active role in ion transport. In connection with this hypothesis, it is of interest that the PLP gene family may have evolved from a pore-forming polypeptide [7]. Thus, myelin may play an active role in ion transport, with respect to not only maintenance of its own structure but also participation in ion buffering near the axon.

20.2.4.6 Receptors Associated with Myelin. Neurotransmitter receptors have been identified on oligodendrocytes and oligodendroglial progenitor cells as well as in compact myelin [56, 57]. A wide variety of these receptors have been found, including acetylcholine (ACh), glutamate, glycine, γ -aminobutyric acid (GABA), dopamine, and β -adrenergic receptors. This field was reviewed in detail [57] resulting in the proposal that proliferation and differentiation of oligodendroglial progenitors can be controlled through the modulation of such receptors. Thus neurotransmitters may play an important role in a neuronal-oligodendroglial communication network. There is evidence that muscarinic receptor activation alters integrin function in oligodendrocytes by modulating binding to extracellular matrix molecules [58]. Neuronal activity influences myelination in opposite ways in the CNS and PNS—promoting it in the CNS and inhibiting it in the PNS [59]. Different purinergic receptors have been shown to have roles in mediating these contrasting effects. Adenosine acts as a potent neuron-glia transmitter to inhibit oligodendroglial progenitor proliferation, stimulate differentiation, and promote the formation of CNS myelin [60]. Conversely, ATP is a signaling molecule in the PNS, where it inhibits Schwann cell proliferation, differentiation, and myelination [59]. With regard to adult pathologies, oligodendrocytes exhibit a high vulnerability to α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) kainate receptor-mediated glutamate excitotoxicity [57].

20.3 DISORDERS OF MYELIN

Deficiencies of myelin can result from a multitude of causes that affect myelin, myelin-forming cells, or myelinated neurons [5, 61]. These include genetic defects, autoimmunity, malnutrition, toxins, viral infections, and mechanical trauma. Disorders of myelin caused by mutations in the genes for myelin constituents were already considered briefly in the above description of myelin composition.

20.3.1 Pathology of Myelinated Axons Related to Disruption of Axon–Glia Interactions

The integrity of myelin sheaths is dependent upon the normal functioning of myelin-forming glia as well as on the viability of the axons that they ensheath. It has long been known that a signal (or signals) from axons to myelin-forming glia is important for the initiation of myelination during development and maintenance of myelin in adults. Many insults to the nervous system initially cause damage to neurons but eventually result in regions of secondary demyelination [5, 61]. The classic model for this is Wallerian degeneration, in which crushing a nerve or myelinated tract results in degeneration of the distal segment. In this process, both axons and myelin eventually disappear due to phagocytosis of debris. Thus any disorder that causes injury to neurons may result in axonal degeneration and lead to the onset of myelin breakdown.

Conversely, the presence of myelin sheaths affects the structure of the axons that are surrounded [62], presumably optimizing their properties for rapid transmission of action potentials. Generally, one of the effects of myelin is to increase axonal diameter by inducing biochemical changes in components of the axonal cytoskeleton such as neurofilaments. The effects of myelin on axonal structure imply that there are signaling mechanisms from myelin or myelin-forming glia to axons. A common theme emerging from recent research on transgenic mice deficient for some of the myelin proteins is that, in addition to their roles in the structure of myelin sheaths, several of them are necessary for maintenance of the axons that are ensheathed. As summarized above, these include PLP, CNP, and MAG, which are expressed by myelin-forming glia but possess important functions necessary for the maintenance and survival of axons.

20.3.2 Autoimmune Demyelinating Diseases

Some of the most significant disorders of myelin are autoimmune in nature and involve inflammatory processes. Multiple sclerosis is the best known of these. Several of the other chapters in this part deal with the neuroimmunological aspects of these diseases in detail. Here, I will briefly discuss some the known and potential roles of myelin proteins and lipids as target antigens in immune-mediated demyelinating diseases.

20.3.2.1 Experimental Allergic Encephalomyelitis. The animal model that most clearly resembles multiple sclerosis (MS) is experimental allergic encephalomyelitis (EAE). It is an acute or chronic demyelinating disease of the CNS which is induced by immunization of susceptible animals with either myelin or various components of

myelin that are encephalitogenic. Detailed examination of the EAE model and the extensive immunological data that have been obtained are beyond the scope of this chapter, and readers are referred to recent reviews for more detail [63, 64]. In brief, EAE can be induced by immunization with several encephalitogenic proteins from myelin, including the major proteins of compact myelin, MBP and PLP. MBP was the first protein shown to cause EAE, and this classical form of the disease is mediated primarily by CD4+ T cells, because it can be transferred from an immunized animal to a naive animal using these cells. The most characteristic component of the EAE lesion is perivascular inflammation, and the extent of demyelination varies between species and the identity of the immunogen used to induce the disease. The amount of demyelination can be enhanced by the presence of antibodies which bind to myelin and provide a ligand for activated monocytes. Indeed, demyelination associated with EAE in several species is increased by the presence of antibodies to surface components of myelin such as galactocerebroside. MOG is specific for the CNS and selectively localized on the outside surface of myelin sheaths and oligodendrocytes [41], making it very accessible to autoimmune attack. Indeed, immunization of animals with MOG alone has been shown to induce a relapsing, remitting demyelinating disease with both cellular and humoral immunity to this glycoprotein [63].

20.3.2.2 Multiple Sclerosis. MS is the most common demyelinating disease of the CNS in humans. The disease primarily affects young adults and is usually characterized by a chronic, highly variable course. The neurological dysfunction often occurs in acute episodes followed by subsequent recovery, and this course is known as relapsing, remitting MS. Over time, the improvement after attacks may be incomplete and the relapsing, remitting course may evolve into one of increasing progression of disability. A few patients have a very aggressive course which can lead to death over a short period.

The cause of MS is not known, but most evidence points to an immunologically mediated disease with important genetic and environmental risk factors [5, 61, 65–67]. The evidence for an autoimmune process is based in part on studies of animal models of autoimmune demyelinating disease such as EAE. Magnetic resonance imaging is providing insights into the natural history of MS and a tool that can be used to monitor new treatments. Blood–brain barrier disruption seems to be the initial event in MS lesions, and pathological studies suggest that enhancement is often characterized by acute inflammation. Based on findings derived from studies of EAE, it is likely that migration of activated T cells represents the initial step in lesion development. However, the antigenic specificity of the T cells is not clear. There are numerous studies of T-cell reactivity to myelin antigens such as MBP, PLP, and MOG, all of which are encephalitogenic in animals. However, the evidence taken as a whole has failed to demonstrate a substantially increased response to these antigens, comparable to that in EAE, in most patients with MS compared to healthy controls. Nevertheless, at this time, MOG appears to be a leading candidate among myelin proteins to be an important target in the autoimmune aspects in many MS patients because of (1) accessibility to immune attack due to its surface localization on oligodendrocytes; (2) its capacity to produce a relapsing, remitting form of EAE resembling MS in animals when used as an immunogen; and (3) the detection of immunoreactivity to MOG in a relatively high proportion of MS patients [63, 68]. It may be that several

myelin antigens play a role in MS and that the relative importance of different antigens varies among individuals depending on their immunogenetic background and environmental factors encountered during their life.

MS lesions or plaques can be identified grossly at autopsy and are sharply demarcated from the surrounding tissue. Microscopic examination characteristically shows loss of myelin with preservation of axons (primary demyelination). However, although the most prominent pathology in MS is demyelination, there have been indications over the years that there is also some axonal pathology. Now techniques of confocal microscopy and immunocytochemistry have clearly demonstrated that transected axons are common in MS lesions [69]. It has become generally accepted that axonal loss is an important aspect of MS pathology and may account for much of the irreversible neurological impairment in this disease. The axonal injury may be caused directly by immune mechanisms, but the loss of trophic support from proteins in degenerating myelin sheaths may also contribute to the pathology. For many years there has been discussion about whether the primary pathological effect causing demyelination in this disease is directed at myelin sheaths themselves or at the myelin-forming oligodendrocytes. Investigators utilizing sophisticated neuropathological and immunocytochemical methods for examining lesions have now reported that there are actually several subtypes of MS patients [70] and myelin sheaths are the primary target in some whereas oligodendrocytes are targeted in others. Most common were cases that resembled EAE in which myelin was the primary target. The pathology was characterized by macrophage/T-cell-mediated or antibody/complement-mediated demyelination. However, other MS patients had lesions characterized by oligodendrocyte dystrophy, which is reminiscent of viral- or toxin-induced demyelination rather than autoimmunity. These patients were less frequent, and lesions of this type were generally observed in patients with a shorter disease course than those with EAE-like lesions. These findings indicate that the demyelinated MS plaque may be a common endpoint resulting from a variety of different immunological and pathological mechanisms.

Unlike MOG, which is exposed on the surface of oligodendrocytes and myelin sheaths, MAG is sequestered at the inside of sheaths and not well situated to be an important target antigen in the initial immunopathological aspects of MS (see Fig. 20.2). Nevertheless, immunocytochemical and quantitative biochemical analyses of myelin proteins have revealed that MAG is often decreased more than other myelin proteins at the periphery of plaques (reviewed in [16]). This may be indicative of early pathological events in the distal periaxonal regions of oligodendroglial processes where MAG is selectively localized. More recent findings suggest that this selective loss of MAG is characteristic of the subset of MS patients that exhibit oligodendroglial dystrophy, and the MAG loss may reflect a dying-back oligodendroglialopathy [70].

20.3.2.3 Autoimmune Demyelinating Disorders of PNS

20.3.2.3.1 Human Autoimmune Neuropathies. Guillain-Barré syndrome (GBS) is an acute inflammatory demyelinating polyneuropathy that is monophasic and self-limiting and frequently occurs following an antecedent bacterial or viral infection [5, 65, 71]. It is generally characterized by primary demyelination, although variants with severe axonal involvement also exist. Chronic inflammatory demyelinating polyneuropathy (CIDP) is similar but is progressive or relapsing and remitting with a

duration of many months or years [5, 65, 72]. With regard to potential target antigens for autoimmune diseases of the PNS, it should be kept in mind that although PNS and CNS myelin are morphologically similar, they differ significantly in chemical composition, especially in protein constituents (see above). Cumulative evidence suggests that nerve injury in GBS and CIDP is mediated by immunological mechanisms, but, as in MS, the role of the patients' cell-mediated and humoral responses in causing the demyelination has not been fully defined. An important role of humoral immunity in these diseases is strongly suggested by findings that sera from GBS patients cause demyelination in appropriate test systems and that plasmapheresis and intravenous administration of immunoglobulin are effective therapies in many of these patients. Neuropathies also occur in association with monoclonal gammopathy (also called paraproteinemia) in which there is expansion of a clone of plasma cells leading to large amounts of a monoclonal antibody in the patient's serum. It is thought that the neuropathies in these patients are likely to be caused by binding of the monoclonal antibodies to neural antigens [73, 74].

20.3.2.3.2 Glycolipids and Glycoproteins as Potential Target Antigens in Human Neuropathies. A substantial body of research now indicates that antibodies to complex glycolipids, such as gangliosides, are involved in the pathogenesis in many of the human immune-mediated neuropathies described above [73, 74]. The first indications of this came from patients with demyelinating sensorimotor neuropathy in association with IgM gammopathy. The monoclonal antibodies in these patients were shown to react with a carbohydrate epitope in MAG that is shared with complex glycolipids and other glycoproteins, including P0 and PMP-22 of compact PNS myelin. The principal antigenic glycolipid, which is present in much larger amounts in the mature PNS than the CNS, is sulfate-3-glucuronyl paragloboside (SGPG). The specificity of these human antibodies is very similar to that of the HNK-1 antibody, which reacts with a number of adhesion proteins in the nervous system. About half of patients with neuropathy in association with IgM gammopathy have antibodies of this specificity. It is also noteworthy that monoclonal IgM antibodies in patients with neuropathy which are MAG/SGPG negative frequently react with gangliosides, suggesting that complex glycolipids may be important target antigens in immune-mediated neuropathies.

Once the monoclonal antibodies reacting with complex glycolipids were shown to occur frequently in patients with neuropathy in association with gammopathy, antibodies were demonstrated in some patients with GBS and other forms of inflammatory demyelinating neuropathy [73, 74]. For example, antibodies to GM1 ganglioside are strongly associated with a subset of GBS that is particularly severe, has a high degree of axonal degeneration, and presents with predominantly motor symptoms. Antibodies to GD1a ganglioside have also been associated frequently with this axonal form of GBS, which is also referred to as acute motor axonal neuropathy (AMAN). Antibodies to GM1 ganglioside also are found frequently in a distinct chronic progressive neuropathy defined by multifocal motor conduction blocks. This condition, known as multifocal motor neuropathy (MMN), is characterized by weakness, muscle atrophy, and motor nerve demyelination while sensory function is normal or only slightly affected. Generally, there appears to be a strong correlation between antibodies to GM1 ganglioside and motor nerve disorders that include GBS, MMN, and the neuropathies in association with monoclonal gammopathy described above. Another correlation of clinical presentation with antibody specificity involves

patients with ataxic neuropathies and antibodies to disialosyl epitopes in gangliosides such as GD1b, GT1b, and GQ1b. This correlation is found in both patients with monoclonal gammopathy and those with the Miller–Fisher variant of GBS.

20.3.2.3.3 Animal Models of Human Autoimmune Neuropathies. Experimental allergic neuritis (EAN), which is caused by immunizing animals with PNS tissue or PNS proteins, is often considered to be a good animal model for GBS. Although the P2 myelin protein is implicated as an important antigen in this model, neither cellular nor humoral immunity to P2 or other myelin proteins has been detected consistently in GBS. Similarly, although antibodies to galactocerebroside have been shown to cause peripheral demyelination in experimental animals, evidence for significant levels of antibodies to this glycolipid in GBS is lacking. Nevertheless, in recent years, there have been major advances in animal modeling in vitro and in vivo of the human neuropathies associated with antiglycolipid antibodies. These results make a strong case that these antibodies cause much of the neurological impairment in the human patients [73, 74]. For example, several animal models involving administration of the human anti-MAG/SGPG antibodies or immunization with SGPG suggest that this disease is caused by the antibodies, but the relative importance of the potential glycolipid and glycoprotein target antigens in contributing to the pathology remains to be established. Furthermore, molecular mimicry between immunogens expressed by antecedent infectious agents and neural gangliosides appears to account for the presence of the glycolipid antibodies in some of the inflammatory polyneuropathies. Thus carbohydrate configurations very similar or identical to those of gangliosides occur in the lipopolysaccharide (LPS) of strains of *Campylobacter jejuni* that are associated with subsets of GBS. Although it is now well established that antiglycolipid antibodies are important pathogenic agents in many patients, not all patients with acquired acute or chronic inflammatory neuropathies express these antibodies. Therefore, much remains to be learned about the pathogenic roles of antibody and T-cell responses to other neural antigens, the importance of cytotoxic immune mediators, and other factors in the acquired immune-mediated neuropathies.

20.4 SUMMARY AND CONCLUSIONS

The composition of compact myelin membranes is novel in comparison to other biological membranes because of its high lipid content and the presence of several major proteins that are relatively specific for myelin. The structural roles of these proteins in compact myelin are rather well understood as summarized in this review. In addition, there are numerous other myelin-related proteins that are not in compact myelin, but rather are present in other associated glial membranes and are important for the normal formation and maintenance of myelinated axons. Some, such as MAG and MOG, have selective localizations in glial membranes within the internodes. MAG is well suited for a role in glia–axon interactions because of its location in the periaxonal glial membrane, whereas the location of MOG on the outside of myelin sheaths renders it a highly accessible target for autoimmune attack. Other myelin-related proteins are located in the paranodal loops, where they have important roles in forming glia–axon junctions and determining the localization of axonal ion channels. As such, aberrations of these paranodal proteins can have

profound effects on the rapid conduction of action potentials. As described in this review, several myelin and myelin-related proteins, including PLP, MAG, and CNP, are expressed by myelin-forming glia but possess important functions necessary for the maintenance and survival of axons. It is now well established that axonal pathology and degeneration are often responsible for much of the neurological impairment in autoimmune and inherited disorders of CNS and PNS myelin [75–77]. One important example is MS, which involves an immune attack on myelin and/or oligodendrocytes but in which much of the irreversible neurological deficit is due to axonal degeneration. The axonal injury in some of these diseases may be caused directly by immune mechanisms, but the loss of trophic support from proteins in degenerating myelin sheaths may also contribute to the pathology.

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PHARMACOLOGY OF INFLAMMATION

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21.1	Introduction	622
21.2	Immune Privilege of CNS	623
21.2.1	Blood–Brain Barrier	624
21.3	Cellular Components of Neuroinflammation	626
21.3.1	Antigen-Presenting Cells	626
21.3.2	Microglia	626
21.3.3	Astrocytes	627
21.3.4	Endothelial Cells	627
21.3.5	Neurons	628
21.3.6	Blood-Derived Inflammatory Cells	628
21.4	Humoral Components of Neuroinflammation	628
21.4.1	Cytokines	628
21.4.2	Chemokines	629
21.4.3	Role of Radical Formation and Oxidative Damage	630
21.4.4	Arachidonic Acid Pathways	630
21.5	Expression of Immunoreceptors in Brain and Their Role	631
21.5.1	MHC Class I and Class II	631
21.5.2	Costimulatory Molecules	631
21.5.3	Toll-Like Receptors	631
21.6	Pharmacological Intervention in Neuroinflammation	632
21.6.1	Activation of Endothelial Cells, Blood–Brain Barrier Opening and Fluid Extravasation, and Cellular Migration to CNS	633
21.6.2	Chemokine- and Chemokine Receptor–Directed Approaches	634
21.6.3	Arachidonic Acid and Prostaglandin Metabolite Pathways	634
21.6.4	cAMP- and cGMP-Modulating Agents	635
21.6.5	Antioxidative Approaches	636
21.6.6	Apoptosis Modulation	637
21.6.7	Modulation of Proinflammatory Pathways (IL-1, TNF- α , Others)	638
21.6.8	Role of Growth Factors (LIF, CNTF, TGF, IGF, Others) in Neuroinflammation	640

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21.6.9	Cannabinoid Receptor Modulation	641
21.6.10	Ion Channel-Directed Approaches	642
21.6.11	Vitamin D Derivatives	643
21.6.12	Statins	643
21.6.13	Adenosine Pathway and Modulation of Purinergic Receptors	644
21.6.14	Indoleamine Deoxygenase and Tryptophan Metabolism	644
21.6.15	Immunization-Based Approaches to Modulate Neuroinflammation	645
21.6.16	Estrogen/Hormones	645
21.6.17	Other Neuroinflammatory Mechanisms and Therapeutic Approaches	646
21.7	Conclusions	646
	References	647

21.1 INTRODUCTION

Inflammation is considered an important pathogenetic aspect in many neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS), but also during hypoxia-induced injury, brain infection, and traumatic brain injury [1–3]. The main characteristics of acute inflammation at peripheral sites—redness (rubor), a sign of increased vascular perfusion, pain (dolor), the presence of nociceptive stimuli, local swelling (tumor), an indication of increased vascular permeability and tissue fluid, and locally elevated temperature (calor)—have been recognized for centuries. The underlying molecular changes, including the upregulation of adhesion molecules and activation of endothelial cells, the extravasation of inflammatory cells and plasma proteins, the tissue activation and proliferation of both immune and local cells, and finally the repair of damage by matrix deposition, represent a common response to all kinds of injury. In acute inflammation, granulocytes predominate, but monocytes and lymphocytes are more characteristic in chronic inflammation. While acute inflammatory responses occur in the central nervous system (CNS) for example, in the meninges during acute meningitis, they are overall much more rare than chronic inflammatory changes that differ substantially from the acute events during neurodegenerative and autoimmune disorders of the CNS. Hence, we will focus on chronic neuroinflammation.

The profound differences in cellular composition and biological characteristics of the CNS in comparison to peripheral organs explain many of the peculiarities of neuroinflammation and its treatment, which will be the subject of this chapter. In the context of immune functions, the eye, testis, and CNS are referred to as immune-privileged sites. What constitutes immune privilege will be detailed below. One important aspect is the almost complete absence of cells of the adaptive or specific immune system such as T and B lymphocytes. Cells that mediate innate immune responses are, however, locally present as microglia, a cell type of the monocyte/macrophage lineage that originates from the bone marrow and represents the only hematopoietic cell in the brain. Activated microglia is the hallmark of chronic neuroinflammation; however, other hematopoietic cells, such as monocytes, granulocytes, eosinophils, and T and B cells, may participate as well depending on the disease type and stage. Furthermore, there is extensive crosstalk between neurons and astroglia on the side of the nervous system and microglia or invading immune cells of the immune system, and signals and mediators from either one can contribute to perpetuating neuroinflammation or to its resolution.

Regarding the unique characteristics of the CNS with respect to inflammation, its immune privilege has already been mentioned. Other factors include the high oxygen metabolism of the brain with the resulting generation of oxygen and nitrogen radicals and at the same time relatively low levels of antioxidative mechanisms, the large amounts of oxidizable polyunsaturated fatty acids in cellular membranes, the very limited capacity of regeneration and proliferation of the few neural precursor cells, and the high metabolic rates of oligodendrocytes and neurons with the resulting susceptibility to insult. Under pathological conditions or normal aging of the CNS, the reduced expression of trophic factors, the dysfunction or overburdening of the ubiquitin–proteasome system with the accumulation of intra- and extracellular protein aggregates, and the elevation of extracellular glutamate and excitotoxicity together with other proapoptotic signals may all contribute to tissue damage and the subsequent activation of microglia and astrocytes. Many inflammatory mediators may in turn sustain and exacerbate degenerative or hypoxic damage, and there is a continuing debate whether inflammatory events precede CNS tissue damage or vice versa. This question is often impossible to resolve; however, currently we assume that there are disease entities where immunological dysfunction and proinflammatory alterations are the primary events, for example in MS [4], while degenerative changes and protein aggregate deposition likely are primary in AD and PD [5]. Even in MS, however, it is becoming increasingly clear that vulnerability of CNS tissue to inflammation, the reduced capacity for repair of damage, and the primary apoptosis of oligodendrocytes in some individuals are important components of the disease process [6]. Furthermore, most of the above diseases are now considered disorders with a complex genetic background; that is, large numbers of susceptibility genes contribute additively and — at the level of the individual gene — weakly to disease expression and course. Depending on whether an individual preferentially harbors genes that predispose to CNS damage [e.g., the apolipoprotein E4 allele (ApoE4) in AD] or genes related to inflammatory insult [e.g., tumor necrosis factor (TNF) alleles] or both, the molecular pathogenesis of the disease processes may differ accordingly.

Finally, it is important to note that inflammation and inflammatory pathways, while often perceived as damaging only in the CNS, are also essential components during tissue repair. Hence, there is increasing interest in delineating which factors are beneficial and how to manipulate them by therapeutic intervention. The duality of inflammation also explains why the simplistic idea that blocking inflammation will always be beneficial is not correct and why a much better understanding of the interactions between immune cells and the nervous system is needed for efficient treatments.

In this chapter, we will discuss the factors that are important for neuroinflammation, such as the CNS immune privilege and the cells and molecular pathways that participate in both damaging effects and repair processes, but also mention the modalities of pharmacological interventions that have either already been tested or are being considered.

21.2 IMMUNE PRIVILEGE OF CNS

Inflammatory components play an important role in many diseases of the CNS. Nonetheless, it is known that inflammatory responses in the CNS differ from

responses in any other tissue. The fact that lymphocytes are virtually undetectable and granulocytes and dendritic cells are absent in the healthy mammalian CNS [7–9] suggests that the CNS is adapted for restricted communication with the immune system and is therefore considered an immunologically privileged tissue. Several factors contribute to the maintenance of CNS immune privilege: (1) the CNS is physically separated from the immune system by the blood–brain barrier (BBB); (2) the low expression of major histocompatibility complex (MHC) molecules in glial and neuronal cells; and (3) the production of anti-inflammatory cytokines such as transforming growth factor (TGF)- β and neurotrophins that maintain the CNS immunosuppressed state [10–12]. However, despite immune privilege, inflammation, either in response to exogenous antigens (infection) or as a result of disrupted peripheral tolerance to self-antigens (autoimmunity), occurs in the CNS.

21.2.1 Blood–Brain Barrier

The BBB exerts a strong restriction on exchange between blood and brain [13–15] that is generally seen as a mechanism for protecting the brain from fluctuations in plasma composition and xenobiotics capable of disturbing neural function. The BBB also plays a role in maintaining the ion homeostasis necessary for neuronal signaling and integration [16].

The BBB is formed by brain endothelial cells that require the acquisition of specific structural and biochemical properties such as the loss of fenestrations, the presence of apical tight junctions, a decrease in pinocytotic vesicles, and the establishment of specific transport mechanisms across the endothelial membrane [13, 14]. In addition, other cell types are associated with the endothelial layer: Pericytes are perivascular cells that form a meshwork on the outer surface and share a basal lamina with the endothelium [17], and the endfeet of perivascular astrocytes ensheath the capillary [18] (Fig. 21.1). The BBB renders brain capillaries 50–100 times tighter than the peripheral microvessels. In spite of the BBB, activated T cells are capable of penetrating it and migrating into the CNS [19, 20], and there is also evidence that antigens can egress from the CNS [21], suggesting that crosstalk between CNS and immune systems may be limited but nevertheless exists.

Additionally, there are a few sites where the BBB is less tight, and there may be preferential migration of cells at these sites. The choroid plexus is composed of a simple epithelium protruding into the ventricles of the brain and delimiting a conjunctive stroma that contains large, fenestrated blood vessels, forming the main barrier between the blood and the cerebrospinal fluid (CSF). The choroid plexus provides potential gates of entry into the CNS for pathogens present in the choroidal stroma during infectious diseases but also for activated lymphocytes, for example, during autoimmune diseases [22–24]. In the choroid plexus, the expression and upregulation of VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) and also the *de novo* expression of mucosal vascular address in cell adhesion molecule 1 (MAdCAM-1) during experimental autoimmune encephalomyelitis (EAE), an animal model of MS, have been demonstrated. Ultrastructural studies revealed polar localization of ICAM-1, VCAM-1, and MAdCAM-1 on the apical surface of choroid plexus epithelial cells and their complete absence on the fenestrated endothelial cells

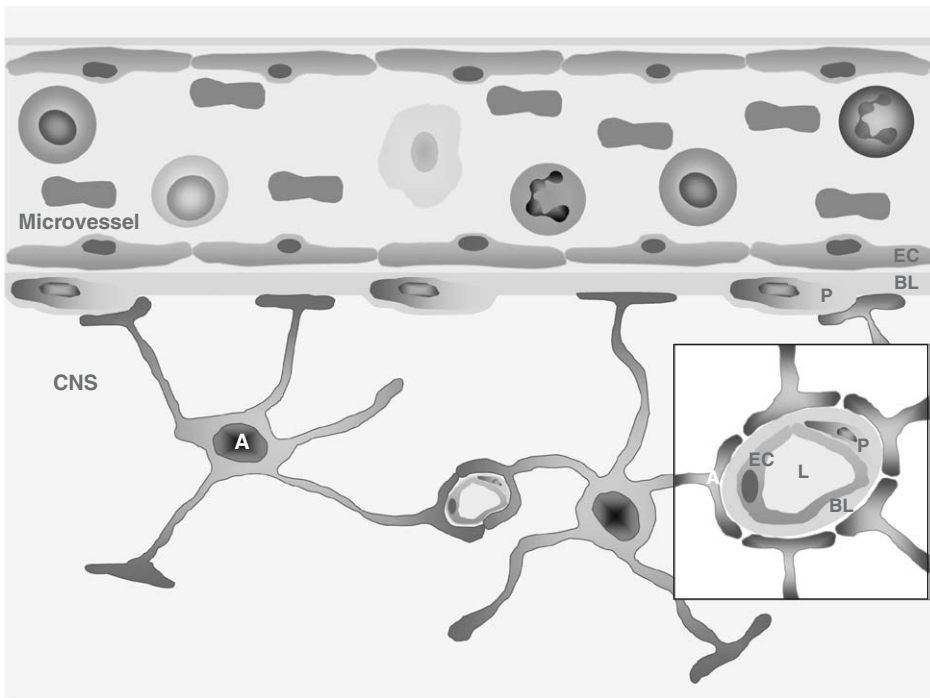


Figure 21.1 Blood–brain–barrier and its components. The interaction of astrocyte (A) foot processes with the basal lamina (BL) and endothelial cells (ECs) is shown in the inset: P, pericyte; L, vessel lumen. (See color insert.)

within the choroid plexus parenchyma. Furthermore, ICAM-1, VCAM-1, and MAdCAM-1 expressed in choroid plexus epithelium mediate binding of lymphocytes via their known ligands. Taken together, these data imply a function of the choroid plexus in the immunosurveillance of the CNS [25]. In the circumventricular organs, which contain neurons specialized for neurosecretion and/or chemosensitivity, the endothelium is leaky. This allows tissue–blood exchange, but since these sites are separated from the rest of the brain by an external glial barrier and from the CSF by a barrier at the ependyma, circumventricular organs do not form a leak or bypass of the BBB [26, 27].

Recently, a number of additional mechanisms have been identified that down-regulate immune reactivity in the CNS and could be considered part of the immune privilege of the brain. Among them are the inhibition of antigen-specific T cells via upregulation of the inhibitory costimulatory molecule cytotoxic T lymphocyte-associated protein 4 (CTLA-4) on astrocytes [28], the downregulation of adhesion molecule and MHC class II expression on microglia by astrocyte-secreted factors including TGF- β [29], and the expression of the tryptophan (trp)–catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), an immunomodulatory pathway that is induced by interferon- γ (IFN- γ) [30, 31]. Different from other immunoprivileged sites, the TNF-related apoptosis-inducing ligand (TRAIL) is not expressed in the brain [32, 33], but invading activated T cells can be eliminated via CD95/CD95L-mediated apoptosis by CD95L-expressing astrocytes [34].

21.3 CELLULAR COMPONENTS OF NEUROINFLAMMATION

21.3.1 Antigen-Presenting Cells

CNS parenchyma is devoid of lymphatic vessels and dendritic cells (DCs) which, in most tissues, endocytose, process, and transport antigens to lymphoid organs to initiate immune responses. Nonetheless, the CSF and interstitial fluid (ISF) of the CNS are now recognized, emphasizing the role of periarterial drainage for the elimination of high-molecular-weight substances from the brain, possibly to regional lymph nodes [35]. Moreover, a role for DCs in the CNS has been demonstrated in MS [36, 37], EAE [38, 39], CNS infections [40, 41], and after injury [42]. In the CNS, DCs can originate from infiltrating blood cells, from choroid plexus and/or meninges [40, 41, 43, 44], or from microglia [39, 42, 45, 46]. In order for T lymphocytes to recognize and respond to antigen, an antigenic peptide has to be processed and presented on the surface of antigen-presenting cell (APC) in the context of MHC [human leukocyte antigen (HLA) in humans] molecules. Potential CNS APCs are endothelial cells, perivascular microglia/macrophages, and, within the parenchyma, the microglia as well as the astroglia, the latter with some restrictions.

21.3.2 Microglia

Microglia are the CNS macrophages with mesodermal origin and share many properties with monocyte-like cells, while neurons and the rest of glial cells differentiate from neuroectoderm [47, 48]. Features common to microglia and systemic macrophages include the expression of innate immune receptors and the ability to phagocytose pathogens, cells, or cellular debris [48–52]. Microglia are very sensitive to changes in the microenvironment of the CNS and become activated when normal neuronal function is disrupted [53]. Microglia activation involves morphological changes, proliferation, and functional differentiation, including upregulation of MHC molecules, secretion of cytokines, chemokines, and nonspecific inflammatory mediators, such as reactive oxygen species (ROS), and become phagocytic at the end stage of activation. Based on histological and immunophenotypic criteria, microglia could be divided into two main types: parenchymal microglia and pericytes or perivascular microglia/macrophages (PVM) located within the BBB. PVM constitute a subpopulation of resident macrophages in the CNS that by virtue of their strategic location at the BBB serve a variety of important functions in both health and disease. They are probably more efficient in fulfilling APC functions compared with parenchymal microglia [54, 55]. In contrast to parenchymal microglia, PVM are frequently repopulated by bone marrow–derived monocytes [7, 47, 56, 57]. It has been described that under nonpathological conditions, both PVM and parenchymal microglia constitutively express high levels of MHC class II [50, 52, 58]. Under inflammatory conditions, parenchymal microglia upregulate MHC class II expression, indicating their participation in antigen presentation. In addition, the B7 costimulatory molecule expression similar to that observed in DCs has been described in microglia [59]. Due to functional and phenotypic similarities with DCs, microglia seem to be capable of initiating and sustaining a T-cell-mediated immune response [51, 55]. The activation of microglia and the subsequent secretion of proinflammatory molecules are pivotal events in the pathogenesis of CNS inflammatory diseases,

including MS [60], AD [61], human immunodeficiency virus (HIV)–associated dementia [62], and intracerebral hemorrhage [63]. Glial activation is part of a defense mechanism to remove debris and pathogens and promote tissue repair. However, inflammatory activation of microglial cells may contribute to the degenerative process through structural invasion and the release of proinflammatory cytokines, ROS, nitric oxide (NO), and excitatory amino acids at synapses and cell bodies. It has been shown that reactive microglia cause neuronal cell death via NO from iNOS, which inhibits neuronal respiration resulting in glutamate release and subsequent excitotoxicity [64]. This may contribute to neuronal cell death in inflammatory, infectious, ischemic, and neurodegenerative diseases.

21.3.3 Astrocytes

The APC function of astrocytes has been extensively investigated. Under normal conditions, astrocytes mainly participate in maintaining normal nerve cell functions by providing a supporting matrix and maintaining the extracellular milieu within the CNS. Astrocytes produce extracellular matrix molecules, clear away diffusing neurotransmitters such as glutamate, regulate ion homeostasis, and produce neurotrophic factors [65, 66]. However, under pathological conditions, astrocytes are activated, become hypertrophic, proliferate, and migrate toward the injury site. Although astroglia do not express MHC class II molecules constitutively, they can be induced by stimulation with the T-cell-derived cytokines such as IFN- γ , TNF- α , or Interleukin-1 β (IL-1 β) [67–69]. In vitro studies have shown that astrocytes exposed to IFN- γ upregulate MHC class II expression and are capable of presenting antigen to T cells [70–74]. In spite of the potential to serve as APCs, conflicting data have been reported on the capacity of astrocytes to activate T lymphocytes [58, 75–78] and on the expression of costimulatory signals and their induction on astrocytes [79–83]. If astrocytes are not competent APCs and fail to fully activate T cells, they may promote T-cell apoptosis or anergy instead. Furthermore, like B lymphocytes, astrocytes may function as nonprofessional APCs by promoting mainly Th2 responses [84, 85]. The fact that astrocyte-mediated regulatory pathways involve the expression of anti-inflammatory mediators TGF- β , IL-10, and prostaglandin E (PGE₂) and the ability to induce the elimination of T cells by apoptosis suggest that they could regulate and limit inflammatory responses within the CNS [86, 87].

21.3.4 Endothelial Cells

Endothelial cells of the BBB are also considered to have some APC functions in the CNS. Due to their strategic anatomical position, they interact with activated lymphocytes entering the CNS. BBB endothelial cells are capable of expressing MHC class II and costimulatory molecules [88], although most available data suggest that endothelial cells are not efficient as professional APCs [89–91]. Like astrocytes, IFN- γ -stimulated brain endothelial cells activate Th2 responses in vitro, whereas they induce only a partial, nonproliferative Th1 differentiation, which might represent a state of relative anergy [92].

Otherwise, during CNS infection or autoimmunity, blood-immune cells such as DCs, monocytes, and B lymphocytes are recruited to the CNS where all of them can contribute to T-cell reactivation.

21.3.5 Neurons

Neurons do not express MHC molecules under physiological circumstances, which protects them from lysis by virus-specific cytotoxic T lymphocytes. Due to their essential functions for the survival of the host organism and the lack of cell proliferation, this absence of MHC expression avoids immune cell-mediated damage, even if a neuron is infected by, for example, a neurotropic virus such as herpes simplex virus. If the functional activity of neurons is compromised, they may, however, express MHC class I and then also become susceptible to T-cell lysis [93, 94]. Neurons engage in extensive crosstalk with immune cells and vice versa via secretion of neurotransmitters, neuropeptides, cytokines, and chemokines during both physiological and pathological conditions. Neurons may induce apoptosis of T cells through CD95–CD95L interaction [95–97], a function which is now also considered part of the CNS immune privilege.

21.3.6 Blood-Derived Inflammatory Cells

The existence of interactions between CNS and immune systems is well established in both the healthy organism and pathological situations, and the CNS is under constant immune surveillance. The inflammatory response includes the participation of different cellular types, including neutrophils, macrophages, mast cells, lymphocytes, dendritic cells, endothelial cells, and others. The recruitment of blood-inflammatory cells to the CNS is mediated by selectins (L-, P-, and E-selectin), vascular cell adhesion molecules (ICAM-1 and -2 and VCAM), and their leucocytic ligands of the integrin family [leukocyte function-associated antigen-1 (LFA-1), integrin alpha M (CD11b) (Mac-1) and very late antigen 4 (VLA-4) [98, 99]. Each of the above inflammatory cells has been found to participate in different states of neuroinflammation or autoimmune damage of the nervous system [4]. For detailed descriptions of their phenotypes and functions the reader is referred to immunology textbooks or specialized reviews.

21.4 HUMORAL COMPONENTS OF NEUROINFLAMMATION

As mentioned above, the exchange of macromolecules and cells between the CNS and the external environment is strictly regulated by the BBB interfaces comprised of cerebral microvessels, the choroid plexus, and the arachnoid membrane. During neuroinflammation and infection, activated immune cells and pathogens can invade the CNS, and immunoregulatory and effector cytokines, chemokines, and other inflammatory modulators such as eicosanoids and matrix metalloproteinases (MMPs) are released into the CSF under these circumstances.

21.4.1 Cytokines

Cytokines are essential mediators of neuroinflammation and neurodegeneration [100–103]. IFN- γ , TNF- α , lymphotoxin- α (LT- α), IL-1, IL-6, IL-8, IL-12, IL-17, IL-18, and IL-23 are important proinflammatory mediators of neuroinflammation in different neuropathological conditions [100, 104–108].

EAE represents probably the best studied model with respect to the pathogenesis of neuroinflammation. In EAE, activated T cells with Th1 cytokine profile have been

identified in the CNS. They secrete proinflammatory cytokines such as TNF- α and IFN- γ [109–111]. TNF- α and IFN- γ secreted by activated T cells activates BBB endothelium and favors leukocyte infiltration [112–115]. IFN- γ is also an important activator of macrophage/microglia, which then contribute to damage by production of NO. NO not only serves a number of physiological role but also is itself neurotoxic and increases BBB permeability [116, 117] and glutamate levels. Increased glutamate in turn mediates excitotoxic damage of both neurons and oligodendrocytes [118].

Another example is IL-1, a potent immune response-generating cytokine expressed by activated microglia. The rapid release of this proinflammatory cytokine is a common feature subsequent to a wide range of CNS injuries [119–121]. Elevated levels of IL-1 have been shown to exacerbate brain damage, while factors that diminish the IL-1 response limit the damage [122–125].

Historically, a detrimental function has been attributed to cytokine-mediated neuroinflammation. However, recently many studies have documented that inflammation and cytokines have a “dual role” in the CNS in that the effects of the same inflammatory mediators may be detrimental, beneficial, or both [126–129]. The balance between these effects is important in determining the outcome.

Another cytokine-mediated mechanism that facilitates local T-cell activation and the perpetuation of immune responses within the CNS is the *de novo* formation of secondary lymphoid tissue [130]. LT- α is an important mediator in this process [130, 131]. Lymphoid structures have been described in the CNS [132–134]. Indirect evidence for the existence of these structures is oligoclonality of the B-cell response in the CNS during MS, which is not observed systemically [135].

21.4.2 Chemokines

Chemokines are a family of proinflammatory cytokines which stimulate target cell-specific directional migration of leukocytes. They have attracted particular interest because of their important roles during inflammation [136–138]. The recruitment of leukocytes implies the presence of chemotactic factors (chemokines), the expression of their receptors, and the expression of adhesion molecules in leukocytes and vascular endothelium. Chemokines are produced upon activation by different cell types such as T lymphocytes, monocytes, endothelial cells, microglia, astrocytes, and neurons [139]. The activity of chemokines may be controlled by proteolytic cleavage, and MMPs are among the proteases that have been implicated in chemokine proteolysis. It has been suggested that chemokine expression is also regulated by IFN- γ , thus controlling the dynamics of leukocyte infiltration into the CNS [140].

Chemokines have been implicated in a variety of CNS diseases and injuries, including HIV-associated dementia, AD, MS, stroke, and head trauma [141–146]. One example is chemokine (C-C motif) ligand 2 (MCP-1) (CCL2), which is produced by astrocytes and represents a key trigger for mediating chemotaxis of monocytes to the injured CNS, since it is a potent activator of macrophage function [147–149]. Different research groups have used the EAE model to study CNS chemokine expression and function [109, 150]. In this context, the temporal and spatial patterns of chemokine expression correlated tightly with the distribution of CNS inflammatory infiltrates in EAE [109] and also with clinical disease activity [151–153]. In MS, it has been recently described that chemokine (C-X-C) motif ligand 12 (SDF-1) (CXCL12) and CXCL13 are elevated in active lesions and CXCL12 also in inactive lesions. CXCL12 may

contribute to axonal damage as it can become a neurotoxic mediator via cleavage by metalloproteases, which are present in MS lesions. CSF CXCL13 levels correlated strongly with intrathecal immunoglobulin production as well as the presence of B cells, plasma blasts, and T cells. These data suggest that CXCL13 is one of the factors that attract and maintain B and T cells in inflamed CNS lesions [154].

The key role of chemokines and their receptors in the pathogenesis of neuroinflammation has rendered them excellent drug targets [155–157]. For detailed reviews of the complexity of the chemokine/chemokine receptor system in the context of neuroinflammation, the reader is referred elsewhere [146].

21.4.3 Role of Radical Formation and Oxidative Damage

NO and other reactive nitrogen species (RNS) appear to play crucial roles in the brain, such as neuromodulation, neurotransmission, and synaptic plasticity, but are also involved in pathological processes such as neurodegeneration and neuroinflammation. Neuroinflammation can be both a cause and a consequence of chronic oxidative stress. Cytokine-stimulated microglia generate large amounts of ROS and RNS, and both neurons and oligodendrocytes are particularly vulnerable to oxidative stress due to their large membrane surfaces and high energy needs. It is now well documented that NO and its toxic metabolite, peroxynitrite, can inhibit components of the mitochondrial respiratory chain leading to cellular energy deficiency and eventually cell death. Oxidants can also stimulate proinflammatory gene transcription in glia, leading to various inflammatory reactions.

21.4.4 Arachidonic Acid Pathways

Various diseases of the CNS are characterized by induction of inflammatory events which involve formation of prostaglandins (PGs). Increases in PG levels are correlated with the development of many inflammatory-related neuropathologies [158–160]. Production of prostaglandins is regulated by activity of phospholipases (PLs) A₂ and cyclooxygenases. The PLA₂ belong to a large family of enzymes involved in the generation of several second messengers that play an important role in signal transduction processes associated with normal brain function. Under normal conditions, PLA₂ isozymes may be involved in neurotransmitter release, long-term potentiation, growth and differentiation, and membrane repair. However, under pathological conditions, high levels of lipid metabolites generated by PLA₂ are involved in neuroinflammation, oxidative stress, and neural cell injury [161].

Neuroinflammation is associated with the production by activated microglia and astrocytes of proinflammatory cytokines such as IL-1 and TNF- α [162, 163]. These cytokines can activate effector enzymes like PLC [164] and PLA₂ [161, 165] and subsequent release of arachidonic acid (AA). Astrocytes are the main CNS cell type that control the release of AA and docosahexaenoic acid and the formation of PGs. The AA cascade is activated under several pathological conditions in the brain such as ischemia and seizures and may be involved in irreversible tissue damage.

On the other hand, AA can exert beneficial effects on brain tissues and cells in several situations. The neurotoxic action is mediated by free radicals generated during AA metabolism, whereas the neurotrophic actions are exerted by AA, PGA₁, and prostaglandin A1 (PDI₂) itself [158, 166].

21.5 EXPRESSION OF IMMUNORECEPTORS IN BRAIN AND THEIR ROLE

21.5.1 MHC Class I and Class II

MHC class I molecules are expressed by almost all nucleated cells of the organism. They serve as the antigen-presenting structure on APCs, since T cells in distinction to antibodies do not recognize antigen in soluble form but only in the context of self-MHC molecules. CD8⁺ cytotoxic T cells are essential to eliminate intracellular pathogens such as viruses and certain bacteria, and they are activated by antigenic peptides in the context of MHC class I. As already mentioned above, the absence of MHC class I on neurons is part of the CNS immune privilege and protects neurons from immune-mediated destruction, for example, during a viral infection [167]. However, neurons have the ability to induce MHC class I molecules [70, 93, 94, 168] and the functionality of these class I molecules has been demonstrated by assays in which neurons are readily killed by cytotoxic CD8⁺ T cells [169]. The ability of microglia, the brain's resident macrophage, to present antigen through the class II MHC to T cells allows these normally quiescent cells to play a critical role in shaping the outcome of many neurological diseases.

21.5.2 Costimulatory Molecules

Costimulation signals are required for full T-cell activation and are assumed to regulate T-cell responses as well as other aspects of the immune system. The families of costimulatory ligands and receptors, CD28/CTLA-4:B7 and CD40:CD40L, are considered critical for full T-cell activation and differentiation into proinflammatory T cells [170, 171].

The CD40 molecule has an important role in promoting inflammatory responses by macrophages/microglia, since interaction with its cognate ligand, CD154 (CD40L), leads to secretion of cytokines and neurotoxins [172, 173]. The expression of CD40 by cerebral endothelium and endothelial cell activation following binding of CD40 to CD40L, expressed in activated CD4⁺ T cells and some CD8⁺ T-cell subsets [174], suggest a potential mechanism by which activated CD40L expressing T cells could enhance adhesion and migration of inflammatory cells across the BBB to sites of inflammation in the human CNS [175].

The B7 molecules CD80 and CD86 expressed on APCs also participate in the costimulation signal required for T-cell activation. B7 molecules bind CD28, which transduces a signal that enhances T-cell proliferation and cytokine secretion, sustains the T-cell response, and prevents the induction of anergy. In neuroinflammatory diseases such as MS, CD80 and CD86 expression are upregulated on activated microglia, infiltrating macrophages [79, 176, 177], and endothelial cells [178].

21.5.3 Toll-Like Receptors

It has long been known that bacterial cell wall products such as lipopolysaccharides but also double-stranded RNA and other molecules can serve as potent monocyte/macrophage-activating signals. In the last few years, specialized receptor molecules, Toll-like receptors (TLRs), have been identified that are highly conserved in

evolution and serve as recognition structures for pathogen-associated molecular patterns (PAMPs). Activation of TLRs initiates the so-called innate responses [179–182]. To date, 10 different TLRs have been identified in humans [183, 184]. In mammals, TLRs bind conserved PAMPs such as lipopolysaccharide (LPS), peptidoglycans, and CpG DNA motifs, which are expressed by pathogenic microorganisms and viruses [185]. TLR-bearing cells are the first line of defense against pathogenic challenge. The TLRs belong to a family of receptors which include receptors for the proinflammatory cytokines IL-1, IL-18, and the receptor T1/ST2 [186]. The cytoplasmic regions of TLR/IL-1R consist of a small globular domain (TIR) which interacts with MyD88 and initiates a signaling cascade that activate Ser/Thr kinases, resulting in the downstream activation of transcription factors such as nuclear factor NF κ B and adaptor-related protein 1 (AP-1) [186] that lead to the transcription of immune and inflammatory genes [187]. TLRs are the main activators of innate immune responses, but they also link the innate with adaptive immunity. Furthermore, it has been proposed that TLRs may have a direct function on adaptive immunity [184]. As one example, TLR2 is expressed on activated and memory T cells and can function as a costimulatory molecule for T-cell activation and to maintain T-cell memory [188].

In the CNS, microglia and astrocytes are capable of responding rapidly to an insult. TLR expression has recently been analyzed in human and murine glial cells, and different TLR expression patterns have been described for microglia and astrocytes. Microglia basally expressed messenger RNA (mRNA) for TLR 1–9, at intermediate levels for TLR 1, 2, 4 and 7, and at low levels for TLR 3, 5, 6, 8, and 9, being in the line with the myeloid lineage. Astrocytes showed a more restricted range of TLR expression. Only TLR3 was expressed at intermediate levels and TLR 1, 4, 5, and 9 at low levels [189, 190].

Astrocytes have been shown to respond to signals of both bacterial (LPS and CpG DNA motifs) and viral infections with increases in proinflammatory cytokine and chemokine expression such as IL-6, IL-12, TNF- α , CCL2 (MCP-1), CCL3 (MIP-1 α), and CCL4 (MIP-1 β) [191–194]. On the other hand, activation of microglia via different TLR stimuli increases expression of proinflammatory cytokines (IL-12, IL-18, and TNF- α) and chemokines [CCL2, CCL3 and, CCL5 (RANTES)] and enhances their APC functions, including MCH class II and costimulatory molecule expression, which enable them to present antigens to CD4+ T cells [195]. Furthermore, it has been described that signals from degenerating axonal terminals can also activate TLRs on glial cells to induce the expression of proinflammatory cytokines and chemokines that drive leukocyte infiltration [103]. These data suggest that glial cells play a key role in both innate and adaptive immune responses, contributing to the inflammatory response in the CNS following an infection or injury.

21.6 PHARMACOLOGICAL INTERVENTION IN NEUROINFLAMMATION

As is evident from the above descriptions of the factors involved in neuroinflammation, a few molecular pathways are particularly relevant, and consequently therapeutic attempts have focused on them. Many details as to how these pathways are interconnected and how immune cells and mediators may influence neurons and

glia and vice versa are either not known at present or are only beginning to emerge. Not surprisingly, therefore, many concepts for therapeutic interventions are based on *in vitro* observations or on animal models and therefore are more theoretic than real in the moment with respect to treating patients. To our knowledge, there is no therapy yet that has been approved with the specific indication to treat neuroinflammation, but a number of anti-inflammatory agents have been tried in AD or other diseases [1, 3, 196, 197]. Below, we will summarize the currently considered modalities to treat neuroinflammation. Potential treatment strategies will be grouped according to the molecular pathways that are involved in neuroinflammation. Data underlying their rationale and known substances/approaches to influence them will be mentioned as well.

21.6.1 Activation of Endothelial Cells, Blood–Brain Barrier Opening and Fluid Extravasation, and Cellular Migration to CNS

Recruitment of inflammatory cells to the site of damage is particularly important during acute inflammation, and the activation of endothelial cells, their upregulation of integrins and adhesion molecules, the local expression of chemokine receptors, and the opening of the BBB with subsequent plasma protein extravasation and also cell migration to the CNS are all interrelated. Steroids at high doses have long been used to interfere with these aspects of early inflammation. They are considered standard treatment during acute spinal cord trauma and MS exacerbations; however, they are not useful during chronic neuroinflammation due to severe side effects. More specific approaches that target, for example, the expression of the adhesion molecule VLA-4 on T cells and monocytes have recently been tested. A monoclonal antibody against VLA-4 proved highly effective in blocking cell migration to the CNS and blocked inflammatory activity by about 80% in relapsing, remitting MS [198]; however, due to either reduced immune surveillance of the brain or increased mobilization of the papovavirus JC from the bone marrow, a few patients developed progressive multifocal leukoencephalitis (PML), an often lethal demyelinating infection of the CNS [199]. Other approaches targeting the adhesion molecules LFA-1 or ICAM-1 either with antibodies, via competition by soluble molecules, or via small molecules either have been tested to block acute but not chronic neuroinflammation or are in preclinical and early clinical development. Since the migration of immune cells into tissues is such an important aspect of maintaining tissue integrity during various injuries, blocking cell migration is associated with substantial risks and therefore likely not a desirable therapy during chronic neuroinflammation.

Opening of the BBB and transmigration of cells also involves matrix-degrading enzymes, in particular a number of MMPs and their tissue inhibitors (TIMPs). MMP-9 has been described as particularly relevant in MS [200]. The ratios of MMP-9/TIMP-1 correlate with magnetic resonance imaging (MRI)–documented CNS disease activity in MS and its reduction also with the treatment effect of IFN- β , which has a profound effect on BBB opening [201]. The hydroxamate MMP inhibitor GM6001 suppressed EAE development and is also therapeutically active in EAE [202]. Besides their role in BBB opening, MMPs are also involved in releasing membrane-bound cytokines or their receptors or activate cytokines from their inactive precursors.

21.6.2 Chemokine- and Chemokine Receptor–Directed Approaches

Comprehensively reviewing the involvement of chemokines and their receptor in immune function and also in nervous–immune system interactions is beyond the scope of this chapter. The reader is referred to specific reviews for this purpose [150]. With respect to neuroinflammation, the CXC chemokines CXCL8 (IL-8), CXCL9 (Mig), and CXCL10 (IP-10) and the CC chemokines CCL2 (MCP-1), CCL3 (MIP-1a), CCL4 (MIP-1b), and CCL5 (Rantes) are of particular interest for the recruitment of inflammatory cells and their local activation [146, 150, 203]. CXCL10 and its receptor CXCR3 are expressed by the CNS and CNS-infiltrating lymphocytes [203]. CCR5 and CXCR3 are markers for proinflammatory Th1 CD4+ lymphocytes [204], and their expression is elevated on peripheral blood and CSF lymphocytes and in CNS lesions in MS [142]. CXCL9–11 are related chemokines, which are all IFN- γ inducible. They are expressed by monocytes, astrocytes, endothelial cells, and fibroblasts and bind to CXCR3 on activated T cells, NK cells, and monocytes/macrophages/microglia. A number of these chemokines (CCL3, CCL5, CXCL9, CXCL10) have been observed in patients with HIV encephalitis [205]. The fact that all of the CXCR3 binding chemokines are expressed in the CNS during neuroinflammatory/autoimmune conditions points to CXCR3 as a particularly attractive drug target. A synthetic CCR5 antagonist (TAK-779) blocks ligand binding to both CCR5 and CXCR3 and inhibits cell migration to the joint in experimental arthritis [206]. Met-RANTES, a peptide that blocks both CCR1 and CCR5, did not inhibit EAE [207]. Various other chemokine receptor antagonists are currently in development or have already reached clinical testing, for example, a CCR1 antagonist in MS. The complexity of the chemokine/chemokine receptor system and the involvement in both damaging and beneficial/repair processes indicate, however, that therapeutic targeting of these pathways will not be straightforward.

21.6.3 Arachidonic Acid and Prostaglandin Metabolite Pathways

Prostaglandins and other AA metabolites play important roles in inflammation. AA is converted to the inflammatory mediators prostaglandins and leukotrienes by prostaglandin synthase and 5-lipoxygenase (5-LO), respectively. 5-LO is upregulated in the brains of MS patients [208]. The rate-limiting enzyme of prostaglandin synthesis, cyclooxygenase (COX), is of particular interest as a target for anti-inflammatory approaches. COX-1 is constitutively expressed, while COX-2 is inducible and rapidly upregulated via mitogens, cytokines, hormones, and phorbol esters. COX-1 is mainly expressed by microglia, COX-2 by neurons, but to a much lower extent also by astrocytes and endothelial cells [209–211]. Neuronal COX-2 expression has recently been found to contribute to glutamate receptor-mediated excitotoxicity via cell cycle deregulation and selective COX-2 inhibitors such as nimesulide-attenuated apoptotic neuronal cell death [212]. Multiple nonsteroidal anti-inflammatory drugs (NSAIDs) have been tested in AD models and also in clinical studies, and the reduction of inflammatory disease mediators in brain tissue has been shown consistently. Clinical testing of NSAIDs that unspecifically inhibit COX-1 and COX-2 suggest a modest beneficial effect and reduction of the risk to develop AD (reviewed in [166]). Selective COX-2 inhibitors have so far not been evaluated in larger clinical trials.

Other potential targets in the above pathways are PLA₂ enzymes. PLA₂ belongs to a large family of enzymes that are involved in generating free fatty acids during lipid peroxidation, among them AA, and lysophospholipids. Several isoenzymes with different intracellular localization, cellular/tissue expression, and activation requirements exist [161]. During normal brain function, PLA₂ participates in neurotransmitter release, long-term potentiation, membrane repair, and cell growth and differentiation. Under pathological conditions such as neuroinflammation, the overexpression of PLA₂ results in membrane phospholipid depletion and alterations of membrane fluidity and ion homeostasis and high levels of free radicals and lipid peroxides [161, 213]. Selective inhibitors of PLA₂ have so far not been developed but might be superior to COX-2 inhibitors.

Prostaglandin E-synthases (PGESs), particularly the membrane-bound isoform mPGES that is functionally coupled to COX-2, have been considered for treatments since both are upregulated in brain endothelial cells following LPS challenge and since mPGES can be upregulated in astrocytes by amyloid beta (A β) [214]. However, no specific mPGES inhibitors are available at present.

Peroxisome proliferator-activated receptor gamma (PPAR γ) represents yet another target. PPAR γ belongs to the steroid/thyroid hormone receptor superfamily and regulates gene transcription together with the retinoid X receptor. The anti-inflammatory prostaglandin PG-J2 as well as NSAIDs may serve as PPAR γ ligand and downregulate macrophage/microglia function via PPAR γ agonism. Findings on the neuroprotective/anti-inflammatory efficacy of NSAIDs in this context could be reproduced with the specific PPAR γ agonist troglitazone, while specific COX-2 inhibition had no effect [215, 216]. PPAR γ agonists are highly effective in the animal model of MS, EAE [217]. The antidiabetic thiazolidinediones, another group of PPAR γ agonists, have been suggested as treatments in AD [218, 219].

21.6.4 cAMP- and cGMP-Modulating Agents

Cyclic adenosine monophosphate and cyclic guanosine monophosphate (cAMP, cGMP) second messengers participate in many different signaling cascades [220]. Eleven families of phosphodiesterases (PDEs) with more than 30 different isoforms are known to date, and these enzymes are involved in regulating the second-messenger levels cAMP and cGMP [221, 222]. Several families and their isoforms are differentially expressed in specific tissues, cells, and different subcellular compartments. Intracellular cGMP levels in the brain are mainly regulated by the highly active calcium/calmodulin-dependent PDE1. Agents that increase intracellular Ca levels decrease cGMP levels that are stimulated by NO donors in both neurons and astrocytes. Another cGMP-specific PDE, PDE5, is found in astrocytes. Both PDE1 and PDE5 are involved in protective mechanisms that prevent excess cGMP signaling under conditions of neuroinflammation, reactive gliosis, and NOS-2 (iNOS) induction.

Cyclic AMP levels are regulated by other PDEs, among them PDE4. PDE4 isoforms are preferentially found in the nervous and immune systems, and different PDE4 isoforms are localized in the cell membrane, cytosol, or nucleus. Inhibition of PDEs results in elevated intracellular cAMP/cGMP levels. In the context of inflammation and neuroprotection, PDE4 inhibitors are of particular interest. The PDE4-specific inhibitor Rolipram was originally developed as an antidepressant [223], but further studies indicated that it is a potent inhibitor of TNF- α / β secretion

[224–226] and IL-12 [227, 228], upregulates IL-4 expression [226], suppresses IL-2 transcription via upregulation of cAMP [229], inhibits B7.1 (CD80) expression, stimulates B7.2 (CD86) expression [230], downregulates proinflammatory cytokines and stimulates Th2 cytokine expression [231], and interferes with proximal steps of T-cell receptor signaling [232]. Furthermore other groups and ours could demonstrate that Rolipram blocks EAE in active-, adoptive transfer-, and chronic-relapsing models in various species, including mice, rats, and marmosets [233–235], but also collagen-induced arthritis [236], adjuvant arthritis, and experimental autoimmune uveitis [237]. Further activities include among others the protection of oligodendrocytes from excitotoxicity [238], an effector mechanism that has recently been shown to lead to oligodendrocyte damage in EAE [118, 239], and the inhibition of HIV replication [240]. PDE4 inhibition has received additional attention due to its effects in promoting axonal outgrowth and functional recovery after spinal cord injury [241–243]. With respect to its influence on CNS function, improvement in synaptic and cognitive function in an Alzheimer's mouse model and the establishment of long-lasting long-term potentiation and improvement of behavioral memory have been demonstrated [244, 245]. Hence, pharmacological intervention via PDE4 inhibition appears very attractive in neuroinflammatory states for a number of reasons. Rolipram is orally available, rapidly enters the CNS, and therefore was considered as a treatment for MS. We conducted a small phase IIa trial at the National Institute of Neurological Disorders and Stroke, National Institutes of Health (Bielekova, Stürzebecher, McFarland, Martin, et al., results unpublished), which was terminated prematurely due to insignificant effect on the primary outcome measure, that is, acute inflammatory CNS lesions. While a well-tolerated dose was established, the known side effects of nausea and emesis developed in some patients. Future therapeutic trials with Rolipram in MS or other neuroinflammatory conditions should incorporate outcome measures that capture effects on neural/myelin repair and neurocognitive improvement. Furthermore, the use of PDE inhibitors that act on two different PDE families is currently being considered. As one example, the combination of PDE4 and PDE3 inhibitors has synergistic effects on lymphomononuclear cells and should broaden the therapeutic window while at the same time lowering side effects that are observed at higher doses of specific PDE inhibitors [230].

Global PDE inhibitors such as pentoxifylline have also been considered in neuroimmunological disorders either alone or in combination with for example, IFN- β in MS [246]. A reduction of proinflammatory cytokines and upregulation of IL-10 secretion and IL-10 mRNA levels were observed in the peripheral blood. Higher doses of global PDE inhibitors are complicated by the cardiovascular effects. Finally, cAMP modulation has been attempted in MS by administration of oral β_2 agonists such as salbutamol. Oral salbutamol in patients with secondary chronic progressive MS reduced the levels of the proinflammatory cytokine IL-12 [247].

21.6.5 Antioxidative Approaches

Neuroinflammatory mechanisms lead to the generation of oxidative stress and damage via a number of molecules, including ROS and RNS, and oxidative damage is considered important in AD, PD, ALS, and MS [1–3, 248, 249]. Radicals are cytotoxic, compromise respiratory chain function, can cause axonal conduction block, release iron from transferrin and ferritin, and together with the impaired

energy metabolism may lead to extensive cell damage. The relatively low glutathione (GSH) levels in oligodendrocyte and neurons further predispose to lipid peroxidation. NO is synthesized from its precursor L-arginine by a number of stringently regulated NOSs with O₂ and NADPH as cosubstrates [250]. Three NOSs are known. NOS-1 and NOS-3 are constitutively expressed by neurons and endothelial cells, respectively, and their activities are stimulated by agents that upregulate intracellular calcium; for example, neuronal NOS-1 is stimulated by glutamate stimulation of *N*-methyl D-aspartate (NMDA) receptors [251]. NOS-2 is inducible by transcriptional regulation and does not require Ca flux. NOS-2 (iNOS) is expressed by monocytes, macrophages, and microglia. The main stimuli of NOS-2 are proinflammatory cytokines and endotoxin LPS; however, β -amyloid peptides, S100 β , and HIV surface proteins have also been shown to induce NOS-2, presumably all via the transcription factor NF κ B [250, 251]. NO gives rise to a number of related radicals, including nitroxyl, nitrous acid, nitrogen dioxide radical, the highly reactive peroxynitrite, and peroxynitrous acid. NO derivatives and NOS-2 levels have been found elevated in AD, PD, MS, and their respective animal models, but despite a wealth of information on their damaging role in demyelination, oligodendrocyte injury, conduction block, and axonal degeneration, their role is far from clear. Besides the detrimental functions, multiple beneficial immunomodulatory roles have been described as well and include the inhibition of antigen presentation and T-cell proliferation, downregulation of adhesion molecules, and direct induction of T-cell apoptosis. Several therapeutic approaches have targeted NO and NOS-2. Aminoguanidine, a partially selective NOS-2 inhibitor, showed benefit in the EAE model; however, its effects strongly depended on the timing of treatment [252]. Another treatment approach in EAE used uric acid (UA) administration [253]. UA functions as a peroxynitrite scavenger, and a beneficial role of UA is further supported by the observation that UA levels in MS patients are usually low and that gout and MS almost never coexist [254]. The multiple roles of NO and NOS in many physiological and pathological conditions indicate, however, that therapeutic manipulation of NO/NOS directly will be difficult, since damaging and beneficial roles often coexist or occur at different concentrations or states of induction.

Antioxidant therapy, for example, by vitamin E, has been shown to reduce microglia activation and AA metabolite production, which in turn lower membrane damage by lipid peroxidation. A large placebo-controlled trial of vitamin E in AD has demonstrated some benefit. Other natural antioxidants such as vitamin C, plant-derived flavonoids such as quercetin [255], and tocopherol derivatives (α -tocopherol phosphate, trolox) have either been found to be effective antioxidants in vitro or have already been tested in a clinical setting [256, 257]. The green tea extract epigallocatechin-3-gallate not only mediates antioxidant activity and chelates iron but also protects brain tissue against NMDA or TRAIL-mediated damage in the EAE model by downregulating cyclin-dependent kinase 4 and reducing NF κ B activation [258].

21.6.6 Apoptosis Modulation

Modulating apoptosis in autoimmune and neuroinflammatory conditions of the CNS may lead to several outcomes. If tissue damage is to a large extent mediated by immigrating autoreactive T cells, then the selective induction of apoptosis of activated T cells in the CNS is a promising goal. Antigen-specific apoptosis or

activation-induced cell death of autoreactive T cells can be induced by exposing already activated T cells to their target antigen again [259, 260]. If one wanted to employ this therapy in vivo, one prerequisite is the knowledge of the relevant target autoantigen. Immunization with a fusion protein of MBP and PLP that encompasses all major encephalitogenic epitopes of these two myelin proteins was successfully used to treat EAE [261], but this approach has not been tested in humans so far. In chronic neuroinflammatory conditions, where invading lymphocytes play a minor, if any, role, apoptosis modulation is more meaningful with the goal of protecting neurons from immune-mediated apoptosis. TRAIL is not constitutively expressed in the human brain; however, both apoptosis-mediating and apoptosis-blocking TRAIL receptors are found on neurons, microglia, and astrocytes [32], and the TRAIL-mediated apoptosis pathway has recently been implicated in neuronal death [262]. The centrally acting analgesic flupirtine-maleate prevents TRAIL-mediated neuronal death and represents an interesting novel neuroprotective approach for states of neuroinflammation [33].

21.6.7 Modulation of Proinflammatory Pathways (IL-1, TNF- α , Others)

A number of cytokines are involved in proinflammatory pathways and neuroinflammation. Among them, the members of the IL-1 and TNF families are particularly important, but IL-6, IFN- γ , granulocyte monocyte colony stimulating factor (GM-CSF), IL-2, IL-12, IL-17, and IL-23 play important roles as well in distinct diseases or pathological states [121, 263, 264].

All ligands [IL-1 α , IL- β , and IL-1 receptor antagonist (IL-1ra)] and receptor components [IL-1RI, IL-1RII, and IL-1 accessory protein (AcP)] are expressed in the brain, but under physiological conditions only at low levels [121]. Caspase 1, which is required for the enzymatic activation of the pro-IL-1 β , is expressed by microglia, and while endothelial cells, astrocytes, oligodendrocytes, and neurons may produce small amounts of IL-1, it is dependent on prior microglia activation. IL-1 is involved in multiple molecular pathways of neuroinflammation and has been found in CNS tissue of many different neuroinflammatory and neurodegenerative conditions (reviewed in [121]). It has therefore been referred to as the “master regulator of neuroinflammation,” and its role has been reviewed extensively elsewhere [121, 265]. To name just a few of its functions, IL-1 may either support or inhibit the growth of astrocytes in different species and it activates the expression of IL-8, M-CSF, G-CSF, GM-CSF, TNF- α , IL-6, CCL5, CCL3, CCL4, NOS-2 (iNOS), and COX-2 in different CNS cells [265]. However, IL-1 also exerts beneficial trophic effects and enhances the survival of neurons via nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and fibroblast growth factor (FGF) induction [266, 267]. BBB repair and production of IGF that supports remyelination are other beneficial effects that are mediated by IL-1-stimulated astrocytes [268]. Furthermore, IL-1 is involved in regulating a number of functions in the normal brain, including sleep, synaptic plasticity, neural transmission, Ca²⁺ signaling, and temperature regulation. From this multitude of effects, one would anticipate that the therapeutic modulation of IL-1 is not an easy task. The natural IL-1 inhibitor, IL-1ra, is now approved for the therapy of rheumatoid arthritis, but it has not been assessed clinically in neuroinflammatory conditions. Since it is a large molecule, BBB permeability will likely pose problems in these disorders. Other approaches include cytokine release inhibitory drugs (CRIDs), which block adenosine

triphosphate (ATP)-induced posttranslational processing of IL-1 β , immunomodulatory cytokines such as IL-10, and cannabinoid receptor ligands (reviewed in [121]).

TNF family members: While IL-1 is considered the primary cytokine of the innate immune response by macrophages/monocytes in response to LPSs, other TLR ligands, and a series of other stimuli, TNF- α and other TNF family members are almost always also involved. The TNF receptor ligand superfamily comprises a large number of cytokines and cytokine receptors including TNF- α , TNF- β (lymphotoxin α ; LT- α), LT- β , the TNF receptors TNFRI (p55) and TNFRII (p75), TRAIL, CD40, CD137, tumor necrosis factor ligand superfamily member 14 (CD258) (LIGHT), and numerous others [269], which cannot all be listed here. In the context of neuroinflammation, we will focus on TNF- α , which is secreted by many cell types, including astrocytes and microglia. TNF- α has been implicated in the pathogenesis of AD, ALS, MS, EAE, and other neuroinflammatory or neuroimmunological conditions [270–272]. Among its potentially pathogenic effector mechanisms are direct toxicity to neurons via TNFRI binding and caspase 3 activation, toxicity to oligodendrocytes, the potentiation of glutamate-mediated excitotoxicity via NMDA receptors [273], the reduction of cAMP levels, increase in ROS and RNS, and others. The damaging effects of TNF- α are accompanied by deficiency of glutathione, a major radical scavenging molecule, which further precipitates cell damage. Besides the well-known proinflammatory, damaging effects of TNF- α , it also has a number of beneficial activities. Besides TGF- β , FGF-2, and platelet-derived growth factor (PDGF), TNF- α levels increase during remyelination [274], and induction of oligodendrocyte progenitor proliferation and remyelination involve TNFRII binding [275]. TNF- α was found to lower IL-18 levels during head trauma [276]. Another TNF family member, LT- α , exacerbates inflammation and demyelination but has no effect during remyelination [277]. Based on the above findings, there is great interest in modulating TNF secretion and activity. In rheumatoid arthritis, TNF-inhibiting strategies by monoclonal anti-TNF antibodies or soluble recombinant TNFR fusion constructs have become standard treatments [278]. When these approaches were tried in MS, disease exacerbations or prolonged relapses and inflammatory CNS activity were observed [279], and these treatments were therefore not pursued further. These unexpected adverse events and disease worsening in a chronic inflammatory disease such as MS highlighted the importance of the dual effects of inflammation and inflammatory mediators. It appears that the activities during remyelination or lesion resolution, which are described above, outweighed the immunomodulatory activities of TNF-targeting approaches.

The potential therapeutic relevance of TRAIL has already been mentioned above.

Other cytokines such as IL-2, IL-17, IL-23, or GM-CSF are important as differentiation or effector molecules in neuroinflammatory conditions. IL-23 as an example is involved in Th1 cell differentiation and acquisition of IL-17 secretion and appears critical for the development of EAE [108]. IL-23 is expressed by microglia/macrophages but also by astrocytes [280]. IL-2, the central growth factor for T lymphocytes that is synthesized by activated CD4+ T cells, crosses the BBB in its natural glycosylated form [281], and IL-2 receptor components have been described on oligodendrocytes, although their function for these cells is only partly known [282]. Similarly, GM-CSF, a proinflammatory hemopoietic growth factor that is important for the differentiation and expansion of granulocytes, monocytes, and dendritic cells, crosses the BBB. GM-CSF and its receptor are also expressed in the brain and involved in both neuroinflammation, and axonal regeneration [264].

21.6.8 Role of Growth Factors (LIF, CNTF, TGF, IGF, Others) in Neuroinflammation

Numerous hematopoietic and CNS growth factors are not only relevant during CNS development and maintenance of CNS homeostasis but also play a role during repair of CNS injury after a variety of different lesions, including stroke, mechanical- and inflammation-induced damage, or tissue deposition of aggregated peptides/proteins that are not cleared by the ubiquitin/proteasome system. Reviewing their physiological roles is beyond the scope of the chapter, and we will only mention some facts that are relevant for the pathogenesis and treatment of neuroinflammatory conditions.

The growth factor/cytokine family that employs the gp130 cytokine receptor subunit includes IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), CNTF, cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC). Some of the members are also referred to as neuropoietins (LIF, CNTF, OSM, IL-6). This group of growth factors/cytokines is involved in inflammatory and immune functions, heart development, fertility, and the survival of neurons and oligodendrocytes [283]. CNTF knockout animals develop more severe EAE and show poor functional recovery with a 60% decrease of oligodendrocyte precursor cells (OPCs) [284]; however, a common null mutation of CNTF in humans is not associated with MS. CNTF was found to inhibit TNF- α -mediated injury. The shared use of the gp130 signaling domain between the above family of cytokines/growth factors explains that IL-6 promotes the survival of dorsal root ganglion (DRG) cells after supplementation with soluble IL-6- α receptor [285]. LIF, another member of this group, exerts a variety of different effects, including growth promotion, cell differentiation of different cell types, influence on bone metabolism, cachexia, embryogenesis, inflammation, and neural development. LIF promotes oligodendrocyte survival after spinal cord injury through augmented expression of insulin-like growth factor 1 (IGF-1) [286] and motor end-plate alterations and loss of distal axons if deleted together with CNTF and/or CT-1 [287]. In the EAE model, LIF directly prevented oligodendrocyte death [288]. However, due to its pleiotropic effects on different cells and differentiation steps, it is difficult to envision at present how to manipulate LIF and LIF signaling therapeutically in human disease.

The biological activity of TGF family members is even more pleiotropic than the above LIF. With respect to neuroinflammation, both proinflammatory and beneficial immunomodulatory effects have been attributed to TGF- β . In AD, PD, and ALS and their models, TGF- β appears to be involved in the neurodegenerative process; however, depending on the experimental paradigm or disease, it is often not straightforward to conclude whether increased TGF- β levels are the cause or effect of the neurodegenerative and neuroinflammatory process. In *in vitro* studies TGF- β together with FGF-2 and PDGF increased the production of myelin basic protein and promoted remyelination [289]. TGF- β also improves the outcome of EAE [290], but a small phase I trial of TGF- β in MS patients had to be stopped because of reduced glomerular filtration rates and kidney function compromise [291].

IGF-1 treatment in a chronic relapsing EAE model led to reduced BBB defects and also reduced demyelination and CNS inflammation. The clinical deficits and lesion severity were improved [292]. Later studies that administered IGF-1 together with IGF binding protein 3 (IGFBP3) showed delayed disease onset but more severe EAE once disease developed [293]. The enhanced expansion of encephalitogenic T

cells was found as the main reason for accentuated disease and highlights again the potential dual effects of hematopoietic factors on CNS cells and vice versa, which are at present not sufficiently understood. Nevertheless, a small phase I/II trial with IGF-1 in MS has been conducted. Treatment was well tolerated, but the trial was not sufficiently sized to detect a therapeutic effect [294].

NGF, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), FGF, and PDGF all have well-documented effects on neurons (NGF, BDNF, GDNF) or oligodendrocytes (FGF, PDGF) and showed beneficial effects in a variety of different experimental systems. In recent experiments in EAE that aimed at preventing and repairing CNS damage by the administration of adult neurospheres, it was observed that the growth factor milieu in the inflammatory lesions contains a number of neurotrophic and gliotrophic regulators that positively influence the treatment outcome [295, 296]. Among these effects were the induction of apoptosis in blood-derived CNS-infiltrating immune cells by neural precursor cells but also the secretion of factors that prevent matrix deposition by astrocytes and glial scarring [295, 296]. While the direct therapeutic administration of individual factors or cocktails of growth factors in the periphery is not feasible and prone to too many side effects, the direct infusion of GDNF into the CNS in PD patients has been attempted and appears well tolerated, but, no positive results have been found thus far. For each of these molecules, delivery into the CNS and ideally into certain areas of the CNS remains a major hurdle. Gene therapy-based approaches, the implantation of cells that secrete growth factors into the CNS such as retinal pigment epithelial cells attached to microcarriers [297], microinfusions, and other special delivery techniques are being explored, but none of these strategies have evolved beyond the experimental stage at this point.

Another growth factor, erythropoietin (EPO), is best known for its effects on erythropoiesis and raising hemoglobin function; however, it was also shown to have cardioprotective and neuroprotective effects in models of stroke, spinal cord compression, diabetic neuropathy, EAE, and optic nerve transection [298–300] via signaling through both Jak2/Stat3 and phosphoinositide-3-kinase (P13K)/Akt [301]. Since EPO appears particularly important in hypoxic preconditioning together with hypoxemia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), there is strong interest to explore its use in stroke, and first clinical trials have begun. One limiting factor is the hemopoietic “side effects” of increased hemoglobin levels; however, recently a number of EPO derivatives have been described that appear to separate the neuro/cytoprotective effects from the ones on erythropoiesis [298].

21.6.9 Cannabinoid Receptor Modulation

Many cell types that are involved in inflammatory reactions express components of the cannabinoid (CB) system [302]. So far, two different CB have been described. CB1 is primarily expressed by neurons, CB2 by immune cells [302]. Following reports about positive effects on spasticity and pain in MS, the therapeutic use of cannabis has been explored recently [303], and a number of laboratories started investigating its molecular mechanism of action on CNS function and in neuroinflammation [302]. There are two endocannabinoids, arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). Plant cannabinoids, notably the main active

compound of cannabis, tetrahydrocannabinol (THC), have been tested in EAE and led to disease prevention when given before antigen inoculation and to delayed disease onset and lower clinical score when given in a therapeutic paradigm [304]. In these early studies, no differences in astroglia and microglia distribution were observed. Later EAE studies focused on the antispastic effects, and THC and synthetic derivatives but not cannabidiol relieved spasticity promptly, and cannabinoid receptor antagonist worsened symptoms [305]. Since CB2 agonist and antagonist appeared to mediate most of these effects, an anti-inflammatory activity was suspected. Later work confirmed that CB2 agonists decreased the expression of IL-1 β , IL-6, and TNF- α [306]. Prolonged EAE in CB1 knockout animals further pointed at a role of CB1 in neuroinflammation as well [307]. With respect to receptor expression, CB1 is found on oligodendrocytes and CB2 on both oligodendrocytes and microglia, and similar to human peripheral blood-derived DCs, microglia upregulate CB2 after stimulation with IFN- γ [302, 308]. Besides the activity in the CNS, CB receptor stimulation leads to apoptosis and decreased proliferation in peripheral blood lymphocytes and inhibition of IL-12, IL-12 receptor expression, and IFN- γ secretion while Th2 cytokine secretion is enhanced. In LPS-synthesized monocytes and macrophages, TNF- α , IL-6, and IL-8 secretion is suppressed (reviewed in [302]). Arachidonic acid and prostaglandin release from monocytes are increased by anandamide [309, 310], and while plant CBs reduce NO production, endogenous CBs enhance it [309]. From these data and recent promising studies in MS, it appears that the use of CB agonists merits further study in neuroinflammation; however, modulation of CB receptors in the periphery and/or the CNS requires a better understanding of the receptor distribution and agonist/antagonist properties and receptor selectivity of individual ligands with respect to specific cell types.

21.6.10 Ion Channel-Directed Approaches

The sharing of a number of cytokine, chemokine, and growth factor receptors between immune cells and cells of the CNS is now well recognized. In contrast, much less is still known about the expression of ion channels and their functional roles on lymphocytes and other immune cells. The expression of various ion channels on T lymphocytes and their potential for immunomodulation have recently gained interest. T-cell activation involves voltage-gated and Ca²⁺-activated potassium channels and Ca²⁺ release-activated Ca²⁺ channels [311]. Numerous small-molecule inhibitors and toxins that block these channels have already been described and are currently being explored in immunological studies [311]. The ion channel-activated signaling mechanisms and how immune mediators modulate them are only beginning to be studied. To give one example, TNF- α modulates T-cell receptor-associated Ca²⁺ signaling via TNFR1-induced sphingomyelinase [312]. Regarding neuroinflammatory and autoimmune diseases, a significantly elevated expression of voltage-gated K⁺ channel Kv1.3 and lower levels of the calcium-activated K⁺ channel IKCa1 were found on effector memory T lymphocytes in MS [313] and also in the brains of MS patients [314]. Inhibition of the same channel on microglia by minocycline was reported to reduce neuronal death in experimental models [315]. Khellinone derivatives [316], peptidic inhibitors [317], psoralene derivatives [318], and phycotoxins [319] are being studied to inhibit Kv1.3 K⁺ and other ion channels

on T cells for immunomodulatory therapies. Administration of the selective intermediate conductance calcium-activated potassium channel protein 1 (KCNN4) K^+ channel blocker TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H pyrazole) protects from EAE by reducing the CNS expression levels of TNF- α and IFN- γ as well as several other proinflammatory mediators [320].

21.6.11 Vitamin D Derivatives

Vitamin D (Vit D) and its derivatives such as calcitriols are well known for their role in regulating calcium metabolism and the growth and differentiation of many cell types. Their immunoregulatory properties are less understood, but downregulation of the NF κ B pathway in T cells and DC and the inhibition of Th1 responses by Vit D receptor (VDR) agonists have recently been demonstrated [321]. Similar to TGF- β , 1 α ,25-dihydroxyvitamin D3 (Vit D3) suppresses IL-12 production and impedes the differentiation of DC, but by different signaling pathways [322]. In the diabetes-prone non-obese diabetic (NOD) mice, Vit D3-suppressed expression of the chemokine IP-10 and of IL-15 did not prevent β cell death but significantly reduced the incidence of diabetes [323]. While the immunomodulatory profile of Vit D3 analogs therefore appears attractive in the treatment of neuroinflammation, there are currently no compounds that dissociate the calcemic properties from effects on the immune system. Before this goal has been achieved, long-term use of Vit D derivatives is difficult to envision.

21.6.12 Statins

Statins reduce low-density lipoprotein and cholesterol levels by limiting the production of mevalonate via blocking 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in de novo cholesterol synthesis. Besides their well-known main indication in atherosclerosis and cardiovascular diseases, statins exert a number of immunomodulatory effects and are of great interest for the treatment of neuroinflammation [324]. Statin-mediated immunomodulation includes the downregulation of LFA-1, the receptor for endothelial ICAM-1, which is important for the transmigration of immune cells from the peripheral blood to the CNS [325]. LFA-1 downmodulation occurs via the Rho family of small GTPases. Transmigration of white blood cells is also affected via the inhibition of MMPs, particularly MMP-9, and furthermore, statins block microglial expression of chemokine receptors CCR5 and CXCR3, which are both considered important for Th1 responses [326]. In EAE studies, it was shown that statins are active in both prophylactic and therapeutic paradigms [262, 327–329]. Their main effect appeared to be the downregulation of MHC class II expression via inhibition of the class II transactivator (CIITA), the shift of Th1 (IL-12 and IFN- γ) differentiation to Th2 cytokine secretion (IL-4 and IL-10) via upregulation of the transcription factor STAT6 (Th2), and at the same time inhibition of STAT4 (Th1) [329]. In a small phase II trial in MS, simvastatin led to an approximately 45% reduction in inflammatory MRI lesions, and hence the use of statins appears very promising for neuroinflammatory conditions in other CNS diseases as well [330].

21.6.13 Adenosine Pathway and Modulation of Purinergic Receptors

It was recently demonstrated that hypoxia induces an immunosuppressive pathway that is mediated via adenosine A_{2A} ($A_{2A}R$)-related mechanisms that lead to the dampening of tissue inflammation and damage [331, 332]. It has long been known that adenosine deaminase (ADA) deficiency and lack of adenosine degradation lead to severe combined immunodeficiency; however, the molecular pathways as to how adenosine leads to immunosuppression have only recently been investigated in more detail [333]. Elevated levels of adenosine and adenosine receptor agonists lead to T-cell apoptosis in the thymus [333], impaired T-cell activation, IL-6 secretion, inhibition of lymphokine-activated killer cell activity, and the TLR-induced upregulation of proinflammatory cytokine expression [334]. The hypoxia in the vicinity of rapidly growing solid tumors or during the adult respiratory distress syndrome (ARDS) and a number of currently used anti-inflammatory/immunosuppressive drugs such as methotrexate and sulfasalazine lead to increased extracellular adenosine and subsequent immunosuppression via $A_{2A}R$. An $A_{2A}R$ agonist (CGS21680) has successfully been used in ARDS [335], and in principle, $A_{2A}R$ agonists and modulation of neuroinflammation via purinergic receptors are attractive. Further support for the relevance of the adenosine receptor pathway comes from studies in A1 adenosine receptor ($A1AR$) null mice, which develop severe progressive relapsing EAE with increased demyelination, axonal injury, and enhanced microglia activation [336]. Treatment with caffeine and the $A1AR$ agonist adenosine amine congener increased $A1AR$ expression on microglia demonstrating that adenosine receptor modulation and activation are a promising treatment strategy in neuroinflammation [336].

Activation of other purinergic receptors, the ATP/P2 receptors, on astrocytes have opposing effects, with $P2 \times 7$ receptor stimulation leading to reduced TNF synthesis following LPS stimulation of astrocytes and activation of the $P2Y$ receptor leading to increased TNF release [337].

21.6.14 Indoleamine Deoxygenase and Tryptophan Metabolism

IDO is an IFN- γ -inducible enzyme that catalyzes the rate-limiting step of the degradation of the essential amino acid tryptophan (Trp) to kynurenine. IDO is expressed by dendritic cells and has been associated with the suppressed immune responses in human cancers, which are associated with elevated Trp catabolism but also with protection from fetal rejection in the human placenta. IDO-expressing plasmacytoid Dcs use the GCN2 kinase pathway in responding T cells to induce anergy [338], but kynurenine breakdown products such as quinolinic acid (QUIN) [339] also lead to T-cell apoptosis via caspase 8 and release of cytochrome *c* from mitochondria [340]. In the CNS, astrocytes, microglia, and neurons express IDO; however, QUIN was only produced by microglia [339]. Hence, IDO may lead to neuronal damage [341], although it was recently shown that an orally active, synthetic derivative of the Trp metabolite anthranilic acid, *N*-(3,4,-demethoxycinnamoyl) anthranilic acid, reversed neurological deficits in the EAE model [342]. Studies with therapeutic approaches aiming at IDO in chronic neuroinflammatory conditions have not been conducted yet.

21.6.15 Immunization-Based Approaches to Modulate Neuroinflammation

Deposition of extra- and intracellular protein aggregates that fail to be degraded through the ubiquitin/proteasome pathway is a hallmark of a number of neurodegenerative disease, including AD, PD, and the tauopathies. In AD, the accumulation of β -amyloid ($A\beta$) peptide aggregates in extracellular amyloid plaques and intracellular neurofibrillary tangles is considered a crucial event in initiating and perpetuating neurodegeneration. The $A\beta$ peptide has the capacity to activate microglia and stimulates many molecular pathways of neuroinflammation such as IL-1 and TNF- α production, and therefore it was considered to stimulate an anti- $A\beta$ peptide immune response by immunizing with the $A\beta_{42}$ peptide itself in order to initiate immune-mediated removal of protein aggregates. This immunization/vaccination approach proved remarkably effective in the transgenic mouse model of AD, in which $A\beta$ overexpression in the brain leads to a similar pathogenetic cascade [343–345]. A vaccination trial in humans that was based on the same rationale had to be halted because approximately 10% of the vaccines developed an acute encephalitis, which was likely caused by the $A\beta$ peptide-specific immune response [346]. It is remarkable that a number of those patients who developed strong immune responses and encephalitis showed a slowed progression or even reversal of their moderate dementia [347, 348]. Data from the animal model in which significant clearing of $A\beta$ aggregates by antibodies and microglia could be shown suggest that such vaccination-based approaches to remove protein aggregates from the brain still hold promise for future therapies; however, we clearly need to understand better how to design such vaccinations in order to render them effective and at the same time safe. Many efforts toward that goal are currently underway, including DNA-based peptide vaccination, the use of recombinant adenovirus vectors that encode for $A\beta$ protein and GM-CSF [349], different administration routes, use of different antigens such as glatirameracetate, and a peptide mixture of the four amino acids A, K, E, and Y [350], which is already an approved treatment in MS. Cell-based immunization approaches have also been proposed. In these, antigen-specific T cells that are transduced with the gene for an immunomodulatory cytokine have been employed successfully in experimental systems [351]. Furthermore, Schwartz and colleagues have demonstrated the beneficial effects of various lymphocyte populations, including autoreactive, proinflammatory T cells in a series of studies of spinal cord injury, optic nerve damage, and EAE [126]. Their work is a particularly striking example that immune mechanisms that have generally been considered damaging also foster repair, and we will hopefully be able to use this knowledge in a systematic fashion for therapies in the future.

21.6.16 Estrogen/Hormones

Estrogen replacement therapy (ERT) in postmenopausal women has shown some benefit in slowing cognitive decline over short periods of time; however, it is not clear whether ERT acts prophylactically. Estrogens and selective estrogen receptor modulation may act either via their effects on neuroplasticity and trophism or via inhibition of microglial activity (iNOS, PGE₂, superoxide release) [352, 353], reduction of microglia [354], and upregulation of proteasome activity [355].

21.6.17 Other Neuroinflammatory Mechanisms and Therapeutic Approaches

Numerous other approaches have been explored for pharmacological modification of neuroinflammation, and these will be briefly listed here.

The tetracycline derivative minocycline was shown to have neuroprotective activity [356] and also to inhibit neuroinflammation and reduce demyelination in EAE [357, 358]. The proposed activities include inhibition of MMPs [358] and microglia activation; however, later studies in a nonimmune model of remyelination showed that macrophage/microglia activation is critical for effective remyelination and that minocycline inhibited remyelination [359]. The combination of IFN- β and minocycline improved the beneficial effects in EAE [360]; however, this may mainly be due to the MMP inhibition via minocycline, which prevents cleavage of IFN- β [361].

Since NMDA receptor-mediated excitotoxicity is involved in neuronal damage via energy depletion and apoptosis induction, it is at least indirectly part of the neuroinflammatory cascade, for example, in AD, stroke, and other conditions. Memantine and other approaches of NMDA receptor inhibition may therefore not only be neuroprotective but also reduce neuroinflammation [362].

Proteinase-activated receptors (PARs) represent a recently discovered group of G-protein-coupled receptors and are widely expressed in neural cells. One of its members (PAR-2) is upregulated on neurons during neuroinflammation in patients with HIV dementia together with increased tissue levels of TNF- α and IL-1 β ; however, the latter was also observed in patients without dementia [363]. The upregulation of PAR-2 expression on neurons protects these cells from HIV Tat-induced neurotoxicity via induction of the tumor suppressor p53 [363].

21.7 CONCLUSIONS

In parallel to a better understanding of the molecular pathways that are involved in neuroinflammation, it has also become clear that the mutual interactions of immune and nervous system cells are extremely complex. The same molecule may exert either proinflammatory or anti-inflammatory effects in either system at different doses, the role of molecules may change depending on their context and the stage/timing of the inflammatory/degenerative process, and besides direct effects on immune/inflammatory cells, immune mediators may be involved in CNS repair and vice versa neurotransmitters or CNS growth factors may be relevant for function and differentiation of immune cells. To give an example, engagement of opioid, vasointestinal peptide (VIP) or adenosine receptors on leukocytes may lead to heterologous desensitization of chemokine receptors, and conversely chemokine signaling may enhance the sensitivity of analgesic opioid receptors on neurons [364]. The immune privilege of the CNS and its relative shielding from the peripheral blood compartment via the BBB complicate treatment strategies that employ macromolecules or even cells. It is obvious from the above, certainly incomplete compilation of therapeutic strategies toward blocking neuroinflammation that there is no lack of ideas. Whether some of these will soon be translated to clinical practice in human diseases will primarily require well-designed and mechanism-of-action-oriented exploratory trials that assess both the safety and efficacy of the various approaches.

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PHARMACOLOGICAL TREATMENT OF MULTIPLE SCLEROSIS

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22.1	Introduction	671
22.2	Treatment of Acute Relapses of MS	672
22.3	Immunomodulatory Therapies for MS	674
22.3.1	Type 1 β -Interferons	674
22.3.2	Glatiramer Acetate	676
22.4	Chemotherapy Agents: Mitoxantrone	676
22.5	Monoclonal Antibodies: Natalizumab and Alemtuzumab	677
22.6	New Proposed Agents for MS: Approved medications with new indications	678
22.7	Conclusions	679
	References	679

22.1 INTRODUCTION

Multiple sclerosis (MS) is a common and often disabling disease of the central nervous system (CNS) [1]. Apart from traumatic injury, it is the most common disabling disease in young adults. It is considered to be primarily an autoimmune and inflammatory disease; however, a degenerative and noninflammatory component appears to be part of the disease and may be responsible for progressive impairment. Most patients begin with a disease course heralded by acute or subacute neurological dysfunction (relapses, attacks) referenced to an area of inflammation within the CNS. The dysfunction typically resolves over days to weeks, but resolution may be incomplete. This disease course is referred to as relapsing, remitting MS (RRMS). A majority of RRMS patients develop progressive neurological impairment that continues to worsen inexorably despite few or no new relapsing inflammatory disease. This stage is termed secondary progressive MS (SPMS). A minority of patients (~10–15%) never have acute clinical relapses and have only progressive neurological impairment. This disease course is termed primary progressive MS (PPMS).

RRMS appears to be primarily due to acute inflammation within the CNS. It is felt that current MS therapies that improve it act primarily as anti-inflammatory agents. The etiology of progressive neurological impairment that occurs with SPMS and PPMS, which appears clinically similar, remains uncertain. A neurodegenerative process of progressive neuronal and axonal loss is felt to be possible but remains unproven [2]. It does appear, however, that current MS therapies do not have a robust effect, if at all, on progressive disease. In fact, none of the medications described below are approved for PPMS (no clinical relapses), as none are proven effective.

This chapter discusses MS therapies aimed at resolution of acute inflammatory relapses of neurological dysfunction and those immunomodulatory therapies used primarily to reduce relapse frequency and severity. The important topic of MS symptomatic therapy is beyond the scope of this chapter and will not be addressed. The aim of immunomodulatory therapies for MS is to limit long-term disability; however, conclusive evidence exists currently only that they may limit short-term disability, lessen relapse rate, and reduce the number of new inflammatory lesions appearing on magnetic resonance imaging (MRI) scans.

22.2 TREATMENT OF ACUTE RELAPSES OF MS

The gold standard treatment of acute relapses of MS is high-dose corticosteroids [3]. Initially, adrenocorticotrophic hormone (ACTH) was used, but this has been widely replaced by high-dose methylprednisolone. Acute relapses of MS are heralded by infiltration of the CNS by inflammatory cells through breaches in the blood–brain barrier. This is visualized radiologically by gadolinium enhancement of demyelinated lesions on MRI of the brain and spinal cord. Corticosteroids are felt to have a beneficial effect by resolving impairment of the blood–brain barrier. This limits infiltration of activated immune cells, including T and B cells, and other accompanying inflammatory mediators into the CNS. Corticosteroids also induce apoptotic death of circulating immune cells that aid their anti-inflammatory actions in improving acute attacks of MS.

One landmark trial that demonstrated the effectiveness of corticosteroids on resolving CNS inflammation was the optic neuritis treatment trial [4]. Optic neuritis, which often occurs in MS, is painful, monocular visual loss that is caused by acute inflammation of the optic nerve. High-dose intravenous corticosteroids were proven to be superior to low-dose oral corticosteroids or oral placebo in hastening visual recovery. Despite the improved rate of visual recovery, there was no demonstration of improved long-term outcome of vision acuity. Initial concern was raised by this trial that low-dose corticosteroids may lead to earlier recurrent CNS inflammation and a definite diagnosis of MS; however, this has not been confirmed subsequently.

Oral very high dose corticosteroids may have a similar effect as that given via the intravenous route [5]. Most centers, however, still prefer a regimen of 3–5 days of methylprednisolone 1 g intravenously once daily with or without a short (5–12-day) oral tapering dose of prednisone (e.g., 60 mg daily reducing by 5 mg every day until discontinued). Many centers do not recommend the tapering prednisone as the short duration of intravenous therapy does not significantly suppress the hypothalamic pituitary adrenal axis.

While the use of corticosteroids for resolution of acute relapses of MS is generally safe, adverse events may occur. Corticosteroid use commonly may cause insomnia and patients may experience irritability. Less commonly, patients suffer frank psychosis or other psychiatric problems. Increased appetite and weight gain often occur, although the effects of this are lessened by the short duration of treatment. There is increased risk for gastrointestinal bleeding and dyspepsia as well as worsened glucose tolerance and exacerbation of diabetes mellitus. Rarely, a severe destructive hip disease caused by avascular necrosis may occur.

As many patients experience substantial recovery with corticosteroids, they remain the gold standard for relapses with associated functional impairment. Patients uncommonly present, however, with very severe relapses of demyelination where severe disability may occur. Some of these patients do not respond satisfactorily to corticosteroids and continue to have severe impairment following treatment. A randomized controlled study demonstrated that, in such patients, a course of seven therapeutic plasma exchanges (TPEs) on an every-other-day basis was effective in regaining functional improvement in approximately 45% of patients [6] (Fig. 22.1). Male patients, treated early, with preserved muscle stretch reflexes may be more likely to improve with plasma exchange [7]. Intravenous immunoglobulin (IVIg) has been useful in other neurological diseases where TPE is effective; however, it remains uncertain whether IVIg improves recovery in similar corticosteroid-unresponsive acute, severe relapses of demyelination.

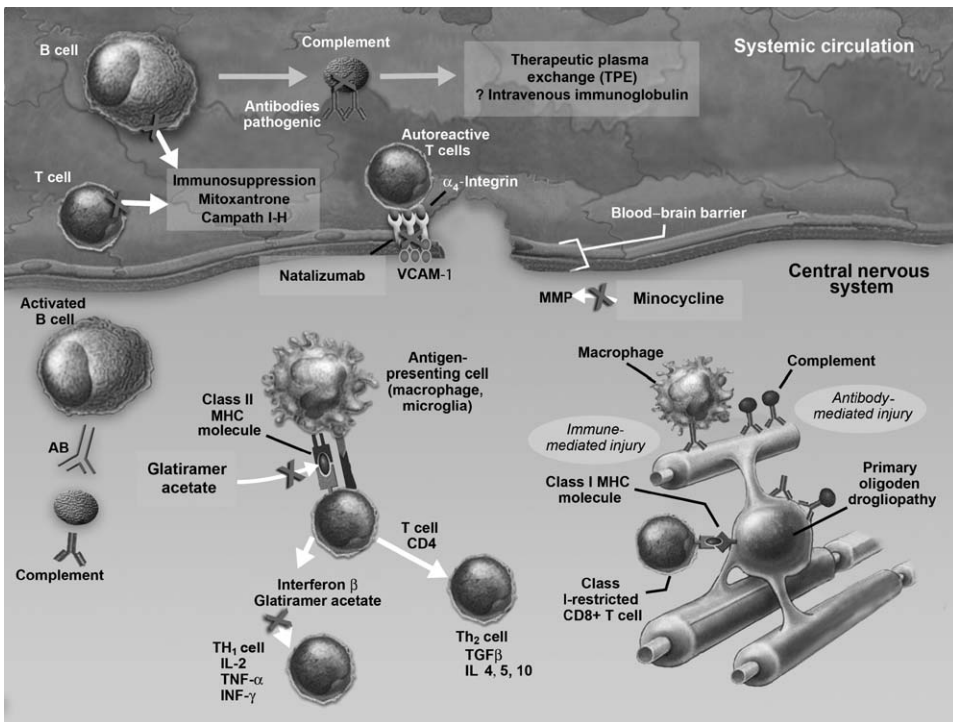


Figure 22.1 AB, antibody; MHC, major histocompatibility complex; VCAM, vascular cell adhesion molecule; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; INF, interferon, MMP, matrix metalloproteinase. (See color insert.)

Actively demyelinating MS lesions are shown to conform to four distinctive pathological subtypes [8]. One of the pathological subtypes is characterized by humoral pathology with evidence of substantial complement activation and immunoglobulin deposition (MS pathological pattern II) [8]. A recent study showed that beneficial response to TPE was highly associated with these humoral pathological changes and MS pathological pattern II [9]. This study revealed that there may be therapeutic importance to the pathological heterogeneity observed in acute lesions of MS. Whether pathological heterogeneity has therapeutic implications with the other immunomodulatory agents apart from TPE is uncertain.

22.3 IMMUNOMODULATORY THERAPIES FOR MS

22.3.1 Type 1 β -Interferons

The first immunomodulatory agent approved for treatment of RRMS was interferon β 1b (Betaseron). Since that approval, two additional therapies of interferon- β 1a have also been approved (Avonex and Rebif). The type 1 β -interferons are proposed to have a number of possible mechanisms of action in reducing attacks and MRI lesions in MS (Fig. 22.1). First, they have been shown to transform a primarily proinflammatory Th1 response to a relatively anti-inflammatory Th2 response (Fig. 22.1). They are felt to have a beneficial effect on the breaches in the blood–brain barrier by inhibiting adhesion molecules and matrix metalloproteinases. They may inhibit T-cell proliferation [10].

The pivotal studies showing clinical and radiological efficacy of interferon- β 1b in RRMS were published in 1993 [11, 12]. These studies showed that in RRMS patients with moderately active inflammatory disease a reduction in clinical attacks of MS by approximately 30% was achieved in the group treated with high-dose (8 million units subcutaneously every other day) interferon- β 1b. There was also a 50% reduction in the most severe relapses. MRI showed a clear reduction in the number of new T₂ weighted and gadolinium-enhancing lesions. A dose effect was clearly demonstrated with more robust reduction in clinical attacks and MRI lesions in the higher dose treatment arm than in the lower dose arm (1.6 million units subcutaneously every other day). Disability was not improved significantly in the treated patients in this study.

Low-dose (6 million units) intramuscular once-weekly interferon- β 1a (Avonex) was approved for RRMS in 1996. A study showed a reduction in short-term disability over 18 months of approximately 35% as measured by the standard expanded disability status score (EDSS) [13]. There was a reduction in clinical relapses of 18%. A reduction in gadolinium-enhancing MRI lesions with this form of interferon- β 1a was also demonstrated.

Interferon- β 1a given subcutaneously three times weekly (Rebif) at both low-dose and higher doses (6 and 12 million units) was shown to be effective in a 1998 study [14]. Acute relapses were reduced by approximately one-third. Favorable improvements were also observed on MRI lesion development, severe relapses, and short-term disability. Despite these results, it was not approved in the United States until a subsequent study showed a greater percentage of patients that were attack free over a short duration, with the high-dose, thrice-weekly, subcutaneous regimen than with low-dose intramuscular, once-weekly dosing [15].

Treatment-related side effects are similar for both interferon- β 1a and interferon- β 1b. Flulike symptoms of malaise, fever, headache and myalgias are very common, particularly early in the treatment course. Injection site reactions with erythema and pain are generally mild and common and are more frequent in the subcutaneous preparations. Significant skin breakdown is fortunately uncommon but is reported and may lead to discontinuation of the medication in some cases. Anemia, leucopenia, and elevation in liver enzymes may occur. Monitoring of complete blood count and liver enzymes is recommended on a regular basis (typically every three months) for the duration of treatment. Rare events of severe liver failure have been reported, and this underscores the importance of regular blood screening. Worsening of depression has been reported but is relatively infrequent as well.

Development of neutralizing antibodies does occur with all preparations of the β - interferons [16]. When persistent, these neutralizing antibodies are associated with a reduction in efficacy of the agents in reducing attacks and MRI lesions. The antibodies are more common with subcutaneous administration and with the interferon- β 1b preparation. Subcutaneous injections of any antigen are known to be more immunogenic. The increased rate of neutralizing antibodies with the interferon- β 1b preparation may be due to the slight difference between it and native human interferon- β (lack of glycosylation, preparation in *Escherichia coli*, and two-amino-acid difference in protein structure). Interferon- β 1a is structurally identical to native human interferon and therefore may be less immunogenic.

Low-dose interferon- β 1a in both subcutaneous and intramuscular preparations has been shown to reduce the occurrence of a second demyelinating relapse in patients with a single CNS inflammatory attack with MRI evidence of prior demyelinating lesions but who do not yet have a definitive diagnosis of MS. This is referred to as a “clinically isolated syndrome” (CIS) of demyelination. Patients with a CIS and an abnormal MRI (two or more typical lesions) are known to be at a very high risk ($\sim 90\%$) of developing another relapse and clinically definite MS over the next 10 years. Two studies showed similar results with low-dose interferons reducing the occurrence of a second MS defining attack from about 50% over 2–3 years with placebo to about 35% with active treatment [17, 18]. These prolongations of time to a second relapse do not, however, appear to reduce the overall likelihood of developing MS in the future, and given the typical relapse reduction, these findings are perhaps not surprising. It has, however, led to approval of low-dose interferon- β 1a in patients with a CIS and evidence of prior demyelination on head MRI.

It does not appear that even high-dose interferons have a particularly robust effect on SPMS and they are not approved for PPMS. Conflicting evidence does, however, exist with a European study showing some benefit in the SPMS population [19]. A concurrent North American study, however, did not confirm the European SPMS findings [20]. It has been observed that the conflicting evidence may have much to do with the patient populations studied. The European patients were found to be more likely to have had evidence of ongoing inflammatory activity with either recent clinical attacks or inflammation noted by new gadolinium-enhancing MRI lesions [21]. Practice recommendations differ; however, some would reserve the use of interferons in SPMS patients with ongoing relapses or substantial accrual of new MRI lesions indicative of new inflammatory disease.

22.3.2 Glatiramer Acetate

Glatiramer acetate (GA; Copaxone) is an engineered medication made of randomly associated L-isomers of four amino acids (glycine, lysine, alanine, and tyrosine). These four amino acids make up the bulk of the major CNS myelin protein known as myelin basic protein. GA is felt to have a number of possible mechanisms of action [22]. Initially it was felt to interfere with antigen presentation via blockade of the major histocompatibility complex (MHC) class II molecule (Fig. 22.1). Like the interferons, it is also felt to drive the immune response away from the relatively proinflammatory Th1 to relatively anti-inflammatory Th2 response.

GA was approved on the basis of a study that showed it decreased MS relapses by 30% compared to placebo [23]. There is no conclusive evidence that shows it decreases disability, particularly over the long term. GA has a positive but delayed effect on reducing new MRI lesions of MS [24]. It has been shown to decrease the degree of T₁-associated hypointensities on MRI. This is felt to be very positive as T₁ hypointense MS lesions are locations of demyelination with particularly significant axonal destruction [25].

GA is administered subcutaneously 20 mg once daily. GA does not have the similar side-effect profile of the interferon-based-medications. It is generally a well-tolerated medication. No ongoing blood tests are needed with GA as it does not cause hematological side effects or elevation of liver enzymes. Patients may develop acute injection-related side effects such as transient chest tightness, shortness of breath, and flushing that do not appear to be serious. Repeated injections may cause some skin breakdown due to lipoatrophy [26].

22.4 CHEMOTHERAPY AGENTS: MITOXANTRONE

Mitoxantrone (Novantrone) is an anthracenedione chemotherapy drug originally approved as an antineoplastic in treating acute nonlymphocytic leukemia (ANLL). It has since also been approved in combination with a corticosteroid for treatment of pain in advanced hormone-refractory prostate carcinoma. The drug intercalates within DNA and inhibits both DNA and RNA synthesis [27]. The mechanism of action in MS is proposed to be its effect on both B-cell and T-cell function (Fig. 22.1). It inhibits B-cell humoral immunity and T-helper-cell function while T-suppressor-cell function is unaffected [28, 29].

Mitoxantrone is structurally similar to the anthracycline antineoplastic agents such as Daunorubicin and Adriamycin. The anthracycline medications have been shown to cause dose-dependent and both reversible and irreversible cardiotoxicity [30]. Cardiotoxicity was evidenced by a reduction in left ventricular ejection fraction and congestive heart failure. Mitoxantrone has also been shown to cause cardiotoxicity. Risk factors that have been identified for the development of mitoxantrone-associated cardiotoxicity in cancer patients are previous use of anthracyclines, mediastinal radiation, and significant preexisting cardiac disease [31]. The risk of mitoxantrone cardiotoxicity is felt to increase significantly at cumulative doses of over 160 mg/m² [31]. Fortunately, cardiotoxicity has been found to be much less common in MS patients as they tend to be younger and healthier and the cumulative dose is less.

Early open studies with mitoxantrone suggested only a mild benefit in progressive MS patients [32]. Further studies with relatively few patients suggested that mitoxantrone was generally well tolerated and of benefit both clinically and in reducing active MRI lesions [33–35]. A recent multicenter placebo-controlled, randomized phase III trial showed significant benefit in a combined group of RRMS and SPMS patients [36]. The investigators found a reduction in progression of disability and clinical relapses with mitoxantrone therapy at doses of 12 mg/m² intravenously every three months over two years. Side effects observed in this study did occur and included mild alopecia, amenorrhea, and urinary tract infections. Leucopenia occurred in 19% of patients treated with 12 mg/m² dosing. Cardiotoxicity appeared to be uncommon and not severe.

The potential for serious side effects from the use of mitoxantrone may limit its use in MS patients. Despite the rare occurrence in the pivotal study, cardiotoxicity has been reported, as have rare hematological malignancies [37, 38]. Currently, perhaps only patients with relatively acute and severe deterioration in functional ability should be considered for this therapy. The patients should be made aware of the potential for serious side effects and be willing to proceed with intensive monitoring aimed at reducing the likelihood of their occurrence.

22.5 MONOCLONAL ANTIBODIES: NATALIZUMAB AND ALEMTUZUMAB

Recently a novel group of monoclonal antibodies has been introduced as possible treatments for MS. The goal of the antibody treatment is to disrupt select important pathways involved in CNS inflammation. One agent was already approved briefly for treatment of MS; however, this was withdrawn voluntarily by the pharmaceutical company only months after approval because of rare, but severe, side effects. This highlights both the potential and pitfalls of engineering new medications for complex diseases such as MS.

Natalizumab (Tysabri) is an inhibitor of lymphocyte trafficking across the vascular endothelium of the CNS blood–brain barrier (Fig. 22.1). It was initially proposed as a therapy for resolution of acute attacks of MS [39]. The medication has a novel mechanism of action. It binds to the α_1 -integrin molecule on circulating T cells. This binding inhibits the interaction between α_1 -integrin on the T cell and its counterpart on the endothelial surface VCAM-1 that is required for infiltration of circulating T cells across the blood–brain barrier and into the CNS.

Natalizumab was shown to effectively decrease relapses in MS [40]. In this study, it decreased attacks 66% versus placebo. Although comparisons between other immunomodulatory agents have not been done, this compared very favorably to the 18–33% reduction compared to placebo of the existing MS medications. More patients remained relapse free on natalizumab than placebo. Gadolinium-enhancing MRI lesions were reduced by over 90% during treatment with natalizumab. Data regarding improvement in disability progression have been encouraging compared to placebo, as well [40a].

Natalizumab is to be given 300 mg intravenously on a once-monthly basis. Treatment is continued indefinitely as patients returned to their baseline frequency of attacks and MRI lesions soon after discontinuation of the medication. Early

studies showed that side effects were relatively minor and uncommon, including headaches, nausea, and fever. Some patients did have an anaphylactic reaction to the medication. This was found to be most frequent on the second to the fourth exposure to natalizumab and appeared to be associated with neutralizing antibody development against the medication.

Natalizumab was voluntarily withdrawn from the market in 2005 when three patients were discovered to have contracted a severe brain infective disease known as progressive multifocal leukoencephalopathy (PML). PML is a CNS white matter disease known to be caused by infection of the oligodendrocytes by the JC virus. It is an opportunistic infection that appears in patients with severe immunosuppression such as infection with HIV or patients on chemotherapy. It is typically fatal but may be treated in HIV patients by aggressive antiretroviral therapy. Both of the initial patients found to have PML had been treated with natalizumab in combination with intramuscular interferon- β 1a. A reexamination of patients treated with natalizumab monotherapy as well as in combination with other agents is ongoing. It remains to be seen at this time whether it will reappear on the market as an agent for the treatment of MS.¹

Alemtuzumab (Campath 1H) is not currently approved for MS. This humanized monoclonal antibody targets CD52 on lymphocytes and monocytes and depletes them within the circulation [41]. Alemtuzumab is given intravenously 20 mg daily for five days typically premedicating with methylprednisolone 1 g on days 1–3. Both CD4 and CD8 T lymphocytes remain depleted for many months following treatment. B lymphocyte counts actually rise and an acute cytokine response is seen. Early studies involved patients with SPMS with gadolinium-enhancing lesions indicative of ongoing inflammatory activity. Studies showed that treatment decreased new MRI lesions and relapses; however, disability was not improved and disease progression continued despite the robust reduction in inflammatory disease [42]. One major concern with alemtuzumab was the occurrence of autoimmune thyroid disease (Graves' disease) in 27% of treated patients [43]. Given its effect on inflammatory MS but not progressive disease, studies are now ongoing to determine whether alemtuzumab is an effective therapy for RRMS.

Daclizumab is a monoclonal antibody against the α chain on the high-affinity interleukin-2 (IL-2) receptor. The IL-2 receptor is primarily responsible for promoting T-cell proliferation and activation. Inhibition of this has been found to be beneficial in reducing renal transplant rejection. Daclizumab is given 1 mg/kg intravenously and then repeated in 14 days and then every 28 days following at a dose between 0.8 and 1.9 mg/kg. Early studies have been promising in RRMS and in some with SPMS [44, 45]. Further studies are planned to confirm or refute these early positive findings.

22.6 NEW PROPOSED AGENTS FOR MS: APPROVED MEDICATIONS WITH NEW INDICATIONS

Statins are acetyl coenzyme-A reductase inhibitors that have revolutionized hyperlipidemia treatment as powerful cholesterol-lowering agents. The statins have also

¹Following Natalizumab was reapproved for use in North America in 2006. In North America it will be monitored by the TOUCH prescribing program as endorsed by the pharmaceutical industries and the Food and Drug Administration.

been found to have significant anti-inflammatory properties [46]. Animal studies have shown promising reduction in a murine model of MS disease of experimental allergic encephalomyelitis (EAE). Human studies are limited currently to a nonrandomized, non-placebo-controlled study that showed a reduction in new MRI lesions after initiation of high-dose simvastatin therapy in patients with RRMS [47]. Pivotal clinical trials are underway to investigate whether the positive findings can be confirmed in a more rigorous scientific trial.

Some tetracycline antibiotics, in particular minocycline, have been shown experimentally to be a potent inhibitor of matrix metalloproteinases, especially MMP-9 [48]. This group of proteinases is responsible for destruction of the basal elements of the extracellular matrix of the blood–brain barrier. This destruction promotes infiltration of activated T cells within the CNS that initiate injury to the myelin. Inhibition of these metalloproteinases is proposed to inhibit CNS injury by this mechanism. A small study showed a promising effect of minocycline on new enhancing MRI lesions [49] and further investigations are continuing on this novel indication as well.

22.7 CONCLUSIONS

Much progress has been made in the pharmacological treatment of MS over the last two decades. Prior to this era, no medications were known conclusively to alter the disease course. Current immunomodulatory medications appear to have most benefit in reducing the inflammatory activity of MS as evidenced by a reduction in clinical relapses as well as new inflammatory MRI lesions. Progressive forms of MS appear much less influenced by these medications, and this suggests an alternative process of neurodegeneration may be responsible apart from inflammation. Advancing knowledge of the mechanisms of demyelination, inflammation, and neurodegeneration is leading to further well-designed studies investigating novel agents that promise to reduce the burden of disease and disability in MS patients.

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23

NOVEL THERAPIES FOR MULTIPLE SCLEROSIS

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23.1	Monoclonal Antibodies	684
23.1.1	Natalizumab	684
23.1.2	Daclizumab	685
23.1.3	Alemtezumab (Campath-1H)	686
23.1.4	Rituximab (Rituxan, MabThera)	687
23.2	New Immunomodulators/Suppressants	687
23.2.1	Statins	687
23.2.2	Temsirolimus (CCI-779)	688
23.2.3	FTY720	689
23.3	Summary	689
	References	689

As currently Food and Drug Administration (FDA)–approved disease-modifying treatments for multiple sclerosis (MS) are only partially effective, the search for new treatment regimens and novel therapies must continue. Currently, patients who fail to respond to first-line disease-modifying therapies, such as interferon- β and glatiramer acetate, are often considered as candidates for nonspecific immunosuppression, reflecting the need for more effective and safe therapeutic approaches. In this chapter we will focus on novel therapies for MS which are being developed with the aim of providing more effective, convenient, and/or specific modulation of the inflammatory component of the disease.

MS is multiphasic. The pathogenesis consists of an inflammatory and a neurodegenerative component. Approximately 85% of individuals begin with relapsing-remitting MS characterized by exacerbations of neurological deficits. After 10 years half of these individuals enter a secondary progressive phase, with persisting and advancing impairment. Another 10% of individuals have a primary progressive course from onset with few, if any, exacerbations. Evidence indicates that the earlier phase of the disease, characterized by distinct attacks with subsequent remission, may be mediated by an autoimmune reaction against the myelin sheath. The subsequent chronic phase is due to degeneration of both the myelin sheath and the

underlying axon. Indeed, it is axonal loss that correlates most strongly with clinical disability [1].

Strategies to improve and enlarge our therapeutic armamentarium have to take into account these different phases or components of the pathogenetic cascade. Current developments focus on systemic immune dysfunction, blood–brain barrier permeability, and the inflammatory process in the central nervous system (CNS). In this chapter we will discuss the potential of newly developed monoclonal antibodies which inhibit recruitment of activated immune cells into the CNS by targeting very distinct cellular molecules. We will also focus on new oral immunomodulators, such as statins, temsirolimus (CCI-779), and FTY720, which are acting on the overactive immune system as the anticipated source of myelin-specific autoimmune cells. Despite these very promising anti-inflammatory approaches, there remains an urgent need for treatments that protect against demyelination and axonal loss or promote remyelination and regeneration.

23.1 MONOCLONAL ANTIBODIES

Monoclonal antibodies are among the most specific novel therapeutic agents designed to target molecules at the immunological synapse. Natalizumab, daclizumab, and alemtzumab, which are currently under investigation for treatment of MS, are humanized monoclonal antibodies. In those antibodies immunogenicity is reduced by grafting of a murine antibody specific for certain human antigens to a human framework resulting in a “humanized antibody.” Rituximab is a chimeric murine/human monoclonal antibody containing human Immunoglobulin G1 (IgG1) heavy-chain and kappa light-chain constant region sequences and murine variable region sequences [2]. Natalizumab, daclizumab, alemtzumab, and rituximab are targeting inflammatory processes in the anticipated autoimmune pathogenesis of MS, such as activation, expansion, and migration of immune cells.

23.1.1 Natalizumab

Natalizumab is a monoclonal antibody targeting the molecule $\alpha_4\beta_1$ -integrin. $\alpha_4\beta_1$ -integrins, are expressed on the surface of activated leukocytes and interact with endothelial cell adhesion molecules (VCAM-1) expressed on inflamed capillary endothelial cells. The resulting adhesion is the first step of a cascade required for migration of leukocytes across endothelia. By blocking $\alpha_4\beta_1$ -integrins, natalizumab inhibits the recruitment of activated leukocytes from the vasculature into the CNS.

When tested in experimental autoimmune encephalomyelitis (EAE), anti- $\alpha_4\beta_1$ -integrin effectively inhibited the accumulation of leukocytes in the CNS and reversed clinical paralysis. In the relapsing, remitting model blockade of $\alpha_4\beta_1$ -integrin effectively prevented further relapses [3]. In a randomized, double-blind trial, patients with relapsing, remitting MS or relapsing secondary progressive MS received intravenous natalizumab or placebo every 28 days for 6 months [4]. There were pronounced reductions in the mean number of new enhancing lesions and relapse rate among patients receiving natalizumab compared with those receiving placebo. However, in this trial natalizumab showed limited activity, with inflammation and

relapses resuming 1 or 2 months after the treatment was stopped, suggesting that continued dosing is required to maintain the beneficial effect. One large randomized, double-blind, placebo-controlled trial tested intravenous natalizumab as monotherapy (AFFIRM study). One-year data of this study showed a 66% reduction of the annual relapse rate with 76% versus 53% of patients remaining relapse free over 1 year. Another large trial tested intravenous natalizumab in MS patients that had experienced one or more relapses while on treatment with Avonex (SENTINEL study). In this add-on study the relapse rate was reduced by 54%. Based on this interim analysis natalizumab was approved by the FDA for treatment of relapsing, remitting MS. However, after one unexpected death and one appearance of progressive multifocal leukoencephalopathy the drug was temporarily removed from the market in February 2005. Progressive multifocal leukoencephalopathy is caused by the polyomavirus JC virus. Most of the primary infections with JC virus occur in childhood. The virus persists in the CNS of the infected individual and is reactivated upon impairment of the immune system, such as human immunodeficiency virus (HIV) infection or neoplastic disease. The decreased migration of leukocytes into the CNS under treatment with natalizumab could impair the balance between immune system and latent JC virus persistence in the CNS. Interestingly, in mice $\alpha_4\beta_1$ -integrin is a cell receptor for murine polyomavirus [5]. Further investigations will have to determine whether specific blockage of $\alpha_4\beta_1$ -integrins or impaired leukocyte recruitment into the CNS in general is responsible for progressive multifocal leukoencephalopathy under natalizumab treatment.

23.1.2 Daclizumab

A central component of inflammation in MS lesions is T lymphocyte activation and the subsequent expansion of T cells mediated by the binding of interleukin-2 (IL-2) to the IL-2 receptor. The IL-2 receptor has low-, intermediate-, and high-affinity forms. The IL-2 receptor α chain (CD25) is only a component of the high-affinity IL-2 receptor but not of the intermediate-affinity IL-2 receptor [6]. The development of a humanized monoclonal antibody specific for the IL-2 receptor α chain (daclizumab) allows to target specifically highly activated T cells. Daclizumab is approved by the FDA for acute renal transplant rejection [7] and has shown promising results in the treatment of autoimmune uveitis [8].

In a small phase II open-label trial with 10 MS patients with either relapsing, remitting or secondary progressive disease course with incomplete response to interferon-beta (IFN- β) and high clinical and paraclinical disease activity add-on treatment with daclizumab resulted in a 78% reduction of new contrast-enhancing lesions and a significant clinical improvement [9]. Another phase II MS study with 19 relapsing, remitting and secondary progressive patients showed sustained efficacy of daclizumab when administered as monotherapy on clinical and magnetic resonance imaging (MRI) disease activity [10]. In both studies daclizumab treatment over 5–25 months was well tolerated; reported side effects included a slight increase of infections during the treatment phase, transient elevations of liver function tests [9], and a rash with response to topical corticosteroids.

An unresolved issue regarding the potential of daclizumab as a future treatment for MS is the fact that it may also nonselectively target regulatory CD4CD25^{hi} cells which can prevent the activation and effector functions of other T cells. A recent

study showed a decreased regulatory function of CD4CD25^{hi} cells in relapsing, remitting MS patients compared to healthy controls [11], raising the possibility that a further impairment of CD4CD25^{hi} cells by daclizumab treatment could contribute to the breakdown of immune tolerance in patients with MS. However, given the positive clinical results and the favorable side-effect profile of daclizumab treatment, larger and placebo-controlled phase III trials are planned.

23.1.3 Alemtuzumab (Campath-1H)

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody against CD52. It targets lymphocytes and monocytes and leads to a sustained depletion of T cells. Campath-1H has been recently licensed by the FDA for treatment of fludarabine-resistant chronic lymphocytic leukemia (CLL). Over the last 15 years it has been explored for transplantation and autoimmune diseases. Particularly, it has shown promising results as treatment for severe refractory autoimmune thrombocytopenic purpura [12] and in rheumatoid arthritis [13].

The mechanisms underlying the very effective alemtuzumab-mediated leucocyte depletion are poorly understood. A single treatment over five consecutive days leads to a sustained depletion of lymphocytes and monocytes. The target of this monoclonal antibody is, compared to other humanized monoclonal antibody therapies, the most widely expressed. Therefore, despite its very distinct antigen recognition alemtuzumab can be considered to be a nonspecific immunosuppressant. The underlying idea is that the T-cell repertoire generated after lymphocyte depletion by Campath-1H would exclude the aberrant autoimmune responses in MS.

Experience from using alemtuzumab for treatment of MS comes mainly from a group in Cambridge, the United Kingdom, which started elective treatment of patients with severe disease course in 1991. By 1999 they treated a total of 36 MS patients with advanced secondary progressive disease course and MRI activity. Radiological and clinical markers were significantly decreased for 18 months after treatment, but disease progression continued [14].

They concluded from the dissociation between decreased inflammatory activity and progressed clinical disability to investigate alemtuzumab treatment at earlier disease stages. Between 1999 and 2004 Coles et al. [15] treated 22 patients with relapsing, remitting MS (RRMS) and observed a 94% reduction of the relapse rate over the following two years after treatment. The average accumulation of disability in this group in the year before treatment [increase of 2.2 expanded disability status score (EDSS) points] compared to -2.4, -0.6, and -0.4 in the periods 0–6, 6–12, and 12–24 months after treatment.

One of the biggest concerns about the safety of alemtuzumab for treatment of MS is the fact that a third of all patients developed antibodies against the thyrotropin receptor and carbimazole-responsive autoimmune hyperthyroidism. Development of Graves' disease was associated with a quicker recovery of CD8 T cells after initial T lymphopenia, which are implicated in the pathogenesis of thyroid autoimmunity [16]. Interestingly Graves' disease had not been reported from any other patient treated with Campath-1H for other disorders, suggesting that patients with MS are uniquely susceptible to this complication.

23.1.4 Rituximab (Rituxan, MabThera)

Rituximab binds specifically to the CD20 antigen, a 35-kDA transmembrane protein, which is involved in cell cycle progression and differentiation of B cells. The CD20 antigen is expressed on normal B cells from pre-B cells to activated B cells but not on differentiated plasma cells, T cells, hemapoetic stem cells, or nonhematopoetic tissues [17]. Rituximab treatment leads to rapid depletion of B cells in the blood. B cells are thought to have an important role in the pathogenesis of MS. B cells, plasma cells, and myelin-specific antibodies are found in large number in active MS lesions [18]. B cells may play a dual role in the pathogenesis of MS as the source for antibody-producing plasma cells and as antigen-presenting cells for activation of myelin-specific T lymphocytes.

Rituximab has shown clear clinical benefit for patients with neoplastic B-cell-mediated diseases [19]. More important for its potential relevance as a future therapy for MS, there is an increasing number of studies showing that depletion of B cells is beneficial in autoimmune diseases with B-cell involvement, such as rheumatoid arthritis [20] and IgM antibody-associated polyneuropathy [21]. Rituximab is generally well tolerated by most patients. The majority of the adverse events that occurred were infusion related. The first infusion may cause a syndrome of fever, chills, and occasional hypotension and dyspnea. Most of the observed severe adverse events were related to preexisting malignant conditions. However, whether the efficacy and safety profile of rituximab in diseases with clear autoimmune B cell involvement can be translated to neurological diseases of the CNS with possible autoimmune B-cell involvement remains unknown. To date, there is only one pilot study with four patients with primary progressive MS investigating the presence and activation status of B cells in the cerebrospinal fluid under rituximab treatment [22]. B cells in the cerebrospinal fluid were not as effectively depleted as their counterparts in the peripheral blood, most likely reflecting the fact that most B cells in the cerebrospinal fluid are CD20-negative plasma cells. The activation status of cerebrospinal fluid B cells, however, was significantly decreased under rituximab treatment. These preliminary findings have to be confirmed in a larger number of patients, and more importantly it has to be elucidated if these effects can ameliorate the clinical disease course of MS. One large clinical trial is underway to evaluate the therapeutic potential of B-cell depletion in primary progressive MS, a smaller trial with test rituximab in relapsing, remitting MS.

23.2 NEW IMMUNOMODULATORS/SUPPRESSANTS

In the following section we will review three promising oral immunomodulators in detail: Statins, temsirolimus (CCI-779), and FTY720. Other agents undergoing phase II/III studies for treatment of MS are Teriflunomide, a *de novo* pyrimidine synthesis inhibitor with antiproliferative activity [23], Xaliproden, which mainly inhibits the synthesis of tumor necrosis factor α [24], chemokine receptor-1 antagonists [25], and mesopram, a phosphodiesterase inhibitor [26].

23.2.1 Statins

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as “statins,” are orally administered cholesterol-lowering drugs. The enzyme

HMG-CoA reductase catalyzes the conversion of HMG-CoA to L-mevalonate. By its inhibition, statins prevent biological activities downstream of L-mevalonate [27]. Currently, statins are the most effective agents available for the treatment of high blood cholesterol levels. In 1987, lovastatin was the first statin to be introduced in the United States, and during this period statins have established themselves as safe and well-tolerated drugs [28–30]. The potential impact of statins on immune function surfaced in 1995, when it was discovered that cardiac transplant patients treated with pravastatin had a reduced incidence of hemodynamically significant rejection episodes and decreased mortality that did not correlate with cholesterol reduction [31]. This landmark observation was followed by a number of studies that have identified immunoregulatory and anti-inflammatory properties of statins.

In EAE, atorvastatin treatment could either inhibit or reverse chronic and relapsing EAE [32]. When treatment was discontinued, only a limited number of experimental animals developed only mild EAE, suggesting a sustained treatment effect [32]. Further investigations elucidated the broad pleiotropic immunomodulatory effects mediated by statin treatment. Statins decreased migration of leukocytes into the CNS, inhibited both major histocompatibility complex (MHC) class II and costimulatory signals required for activation of T cells, and decreased proliferation of proinflammatory T lymphocytes and expression of inflammatory mediators in the CNS [32].

Simvastatin has been tested in a small open-label trial of patients with clinically definite relapsing, remitting MS using a crossover design [33]. Thirty MS patients received monthly brain MRIs in a pretreatment period of three months, followed by six months of treatment with 80 mg simvastatin daily, the highest FDA-approved dose. They received brain MRIs at months 4, 5, and 6 of simvastatin treatment. Analysis of pre- and posttreatment MRI data indicated a decrease of approximately 45% in the mean number of gadolinium (Gd)-enhancing lesions and in the mean volume of Gd-enhancing lesions in treated subjects. A multicenter placebo-controlled trial, which is currently under enrollment, will evaluate whether treatment with 80 mg atorvastatin can reduce the risk of further MS activity in patients that have experienced their first demyelinating attack, a “clinically isolated syndrome.”

23.2.2 Temsirolimus (CCI-779)

Temsirolimus is an ester analog of sirolimus. Temsirolimus binds to a cytosolic protein, FKBP12, which subsequently inhibits the mammalian target of rapamycin (mTOR) [34]. Inhibition of mTOR blocks a number of signal transduction pathways that suppress translation of several key proteins regulating the cell cycle. These effects lead to a cell cycle block in the G₁ phase. In animal models of human cancer, temsirolimus inhibited the growth and diverse range of cancer types even when an intermittent dosing schedule was used. Temsirolimus has the potential to block the inflammatory responses related to autoimmune diseases by blocking T-cell proliferation. Furthermore, through its properties as a neuroimmunophilin ligand, CCI-779 may also have neuroprotective and/or neuroregenerative activities.

In August 2004 temsirolimus received fast-track status from the FDA for the first-line treatment of poor-prognosis patients with advanced renal cell carcinoma. Based on the mTOR mechanism of action, temsirolimus is in clinical trials to investigate its therapeutic utility in autoimmune diseases like MS and rheumatoid arthritis.

23.2.3 FTY720

FTY720 is the first oral immunosuppressant with a unique mode of action. Structurally similar to natural sphingolipids, it inhibits T-cell recirculation by activating sphingosine 1-phosphate G-protein-coupled receptors, which in turn leads to the increased sequestering of T cells into the lymph nodes. FTY720 has no effect on T-cell activation or expansion at clinically relevant doses. Its ability to penetrate the blood–brain barrier makes it an attractive candidate for treatment of MS.

Japanese researchers have recently investigated the efficacy and mechanism of immunosuppression produced by FTY720 in EAE [35]. FTY720 treatment almost completely protected against disease in this model. The protection by FTY720 was associated with a dramatic reduction in the number of lymphocytes staining for T-cell receptors in the spinal cord. The messenger RNA (mRNA) expression of Th1 cytokines in the spinal cord was also reduced dramatically. Furthermore, adoptively transferred lymphocytes isolated from the spleen of FTY720-treated rats reduced both disease incidence and clinical score in recipient untreated animals with EAE. These results suggested that the protective anti-inflammatory effect of treatment with FTY720 was, to a large extent, due to the inhibition of encephalitogenic T-cell responses and/or their migration into the CNS.

FTY720 is currently under investigation for treatment of relapsing-remitting MS and results are anticipated with high expectations.

23.3 SUMMARY

Currently FDA-approved drugs for MS are partially effective and administered parenterally. In order to improve therapy for MS new developments focus on specific inflammatory aspects in MS pathogenesis. A variety of monoclonal antibodies targeting distinct molecules on the surface of immune cells have been developed. They are inhibiting expansion, activation and /or migration of immune cells into the CNS. Despite their specificity those potent drugs have to be tested carefully. Natalizumab, which was the first of these monoclonal antibodies to be approved for MS therapy was temporarily withdrawn from the market following a case of progressive multifocal leukoencephalopathy under treatment. Another aspect how to improve MS therapy is to develop oral immunomodulators. Those drugs, targeting the overactive immune system, are not only more convenient to administer. They are also excellent candidates to be given in combination, which might be a valuable therapeutic approach in addition to the search for more effective mono-drug therapies.

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24

NEUROPHARMACOLOGY OF HIV/AIDS

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24.1	Introduction	694
24.2	HIV/AIDS of Nervous System	695
24.2.1	Pathogenesis of HIV-Related Neurological Diseases	696
24.2.1.1	Chemokines in HIV-Related Neurological Diseases	697
24.2.1.2	Mechanisms of Neurodegeneration in HIV-Related Diseases	699
24.2.2	Neurotoxic HIV Proteins	699
24.2.2.1	Structural Glycoproteins gp120 and gp41	699
24.2.2.2	Non-Structural HIV Proteins	701
24.3	Cell Death Cascades in HIV-related Neurological Disease	704
24.3.1	Apoptosis	704
24.3.2	Excitotoxicity Cell Death	705
24.3.3	Oxidative Stress	706
24.3.4	Effects of HIV on Neural Progenitor Cells	706
24.4	Alterations in Lipid Metabolism in HIV	707
24.5	Nigrostriatal System in HIV/AIDS	707
24.5.1	Response to DA Mediators	709
24.6	Other Neurotransmitter Systems in HIV-Associated Dementia	711
24.6.1	Excitatory Amino Acid Neurotransmitters in HAD	711
24.6.2	Cholinergic System in HAD	711
24.7	Drugs of Abuse in HIV/AIDS	712
24.7.1	HIV and Methamphetamine/Cocaine	713
24.7.2	Opioid Drugs	715
24.7.3	Alcohol	716
24.8	Neurodegenerative Diseases in HIV/AIDS	716
24.9	Conclusion	718
	References	719

24.1 INTRODUCTION

Neurological diseases have been a prominent feature of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) since the pandemic began. The advent of highly active antiretroviral therapy (HAART) resulted in a significant reduction in the opportunistic infections and cancers that were common early in the pandemic. Unfortunately, although patients are living longer, HIV-related neurological disease continues to have a significant impact on patients with HIV/AIDS [1]. HIV encephalitis (HIVE), HIV-associated dementia (HAD), and mild cognitive motor disorder (MCMD) continue to be seen in patients with HIV/AIDS. The continued impact of HIV on the nervous system is thought to result from continued low levels of HIV replication that results from poor penetration of antiretroviral drugs into the brain.

HIV infection in the brain is found primarily in monocytes, macrophages, and microglial cells [2]. Limited HIV replication occurs in astrocytes while oligodendrocytes appear to be spared. HIV infection of neurons is rare despite the presence of significant neuronal cell death in patients with HIV-related neurological disease. These findings suggest altered neural function and cell death may be the result of neurotoxic effects of HIV-encoded proteins, proinflammatory molecules, or both.

The neuropharmacological features of HIV infection are central to understanding the neurological diseases encountered in HIV/AIDS (Table 24.1). HIV infection occurs predominantly in the basal ganglia. The clinical features seen in HIV-infected patients with neurological disease have many similarities to those found in extra-pyramidal disorders such as Parkinson’s disease (PD), Wilson’s disease, and others [3]. Dopamine metabolism and levels are altered in HIV-infected brains while virus-encoded proteins are toxic to dopaminergic neurons. HIV regulatory and structural proteins can alter functions of neurotransmitter receptor functions and several, at least experimentally, can form ion channels in cell membranes. Dopamine and drugs of abuse can act synergistically as neurotoxins with HIV-encoded proteins.

The prolonged survival of patients with HIV/AIDS on HAART has shifted the prevalence of HIV-related neurological diseases to older age groups [1]. The older HIV/AIDS patient will be approaching a time when they would otherwise be at risk for Alzheimer’s disease (AD), PD, and other neurodegenerative disorders. It is conceivable that these patients, even without HIV-related neurological disease, will

TABLE 24.1 Neuropharmacological Features of HIV-Associated Diseases

Basal ganglia and its neural circuits are primary targets of HIV infection.
Neurotransmitters and receptors are involved in neurotoxic pathways leading to neurological disease.
Dopamine, dopamine agonists, and drugs of abuse that act through dopaminergic system facilitate HIV replication and accelerate HIV-associated neurological diseases.
Pharmacological synergy between HIV-encoded proteins and neurotransmitters and drugs amplifies neurotoxic effects in the brain.
Cell death cascades encountered in neurodegenerative diseases are central in the pathogenesis of HIV-associated neurological disease.
HIV alters neural progenitors affecting neural circuits.
Lipid boats are disrupted by HIV-encoded proteins.

be at increased risk for developing neurodegenerative diseases which could be more severe and begin at an earlier age as a result of prior damage to basal ganglia and other neuropharmacological systems. Complicating an already complex situation, drugs used to treat neurodegenerative diseases may potentiate HIV-related effects on these disorders.

The neuropathogenesis of HIV/AIDS, the effects of drugs of abuse as well as therapeutic agents to upregulate HIV expression and accelerate HIV-related diseases of the brain, and the possible interactions of HIV with other neurodegenerative diseases as HIV/AIDS patients live longer emphasize the importance of neuropharmacology in understanding and treating patients with HIV/AIDS.

24.2 HIV/AIDS OF NERVOUS SYSTEM

Between 30 and 40% of individuals infected with HIV will develop a range of cognitive and motor symptoms in the developed world [4] (Table 24.2). The neurological diseases associated with HIV include HAD, MCMD, vacuolar myelopathy, and distal sensorimotor neuropathy. Patients with HAD develop a subcortical dementia with cognitive slowing, impaired attention span, short-term memory impairment associated with motor signs attributable to the basal ganglia including slowed alternating movements, bradykinesia, reduced facial expression, and postural imbalance. Weakness in the legs has been attributed to an associated vacuolar myelopathy. Behavioral changes are common and include alterations in personality, apathy, and social withdrawal. Terminally, patients with HAD develop a vegetative state commonly seen in dementias.

The incidence of HAD has declined in patients treated with HAART [5]. HAART has also resulted in an increased survival of patients with HIV/AIDS. As patients live longer on HAART, the prevalence of HAD has increased despite the reduction in incidence of dementia [6]. A milder form of cognitive and motor impairment classified as MCMD has been recognized during the HAART era. Whether MCMD has become more common because of HAART or is more clinically apparent because patients are not progressing to HAD is unknown. One possible explanation for MCMD is the poor penetration of antiretrovirals, especially protease inhibitors, into the brain allows for low-level virus replication [4]. It is not known

TABLE 24.2 Pathogenesis of HIV-Associated Disease

HIV infection occurs predominantly in mononuclear cells in the brain; astrocytes are infected, neurons rarely infected.
Immune response is out of proportion to level of virus infection, leading to high levels of proinflammatory molecules.
Neuronal dysfunction and death follow from the neurotoxic effects of viral proteins, neuromodulatory molecules, and dopaminergic transmitters.
Alterations in astrocyte function are directed at maintaining neurons and extracellular milieu by HIV infection.
Neurotoxic pathways converge on cell death cascades, including apoptosis, oxidative stress, and mitochondrial dysfunction similar to that seen in Alzheimer's disease, Parkinson's disease, and other neurodegenerative disorders.

whether patients with MCMD are at risk of progressing to HAD. The fact that MCMD is associated with an overall worse prognosis for survival of HIV-infected patients suggests it will be important to further define the natural history of MCMD and what, if any, factors place patients at risk for further cognitive decline.

Current estimates suggest approximately 10% of HIV-infected adult patients have HAD with up to 30% exhibiting symptoms and signs of MCMD [5]. Both HAD and MCMD are associated with neuropathological changes consistent with HIV infection of the brain. The severity of HIV infection present in the brain does not correlate with the degree of cognitive and motor impairment. Patients with severe HIVE may have only minimal cognitive changes while others with minimal neuropathological changes attributable to HIV infection nonetheless have severe dementia. The reasons for disparity between the degree of HIV infection and severity of neurological disease are complex and not completely understood. As will be seen, the molecular events leading to neurological disease encompass both viral-encoded proteins and proinflammatory molecules that may act synergistically, making correlation of neuropathological features with clinical disease difficult.

24.2.1 Pathogenesis of HIV-Related Neurological Diseases

HIV infection of the brain occurs shortly after primary infection [2]. Several routes have been proposed for HIV entry into the brain [4]. HIV-infected mononuclear cells provide the principal pathway for HIV to reach the brain. Although HIV-infected lymphocytes cross the blood–brain barrier (BBB), they likely have only a minor role in the pathogenesis of HIV infection of the brain. Cell-free HIV may enter the brain by first infecting cerebrovascular endothelial cells with release of virus into the perivascular space. Perivascular macrophages, microglia, and astrocytes may then become infected by HIV that has crossed the BBB via endothelial cells. HIV may also reach the cerebrospinal fluid (CSF) compartment by trafficking of HIV-infected mononuclear cells into the CSF or infection of cells of the choroid plexus. The importance of the monocyte/macrophage cell lineage in transporting HIV into the brain is further supported by the consistent isolation of macrophage trophic HIV strains from brain [2, 7, 8]. T lymphocyte trophic HIV strains are rarely isolated from brain tissue.

It is unclear whether HIV remains in the central nervous system (CNS) throughout the course of infection or is cleared by the immune system only to reenter the brain at a later date. Phylogenetic analysis of brain shows that HIV isolates are more closely related to each other than they are to HIV isolates from other sites in the same patient [2, 4]. It has not been conclusively established whether these differences reflect a founder effect from different isolates entering the brain at different times or reflect specific adaptations of HIV to replication in the brain. The reduced immune responses to HIV infection in the brain, including lower neutralization antibody titers and T-cell surveillance, may facilitate HIV replication in the brain compartment leading to divergence of viral sequences within the brain compartment compared to systemic isolates. HAART-related limitation of HIV replication in the brain could also reduce the effects of genetic drift in the viral population of the brain compared to that seen in peripheral organs.

HIV replication in the CNS occurs principally in cells of the monocyte/macrophage lineage [7]. Subtypes of macrophages and microglia are classified by location,

morphology, and specific cell surface markers. Perivascular macrophages as well as microglia and astrocytes in the perivascular region come into direct contact with HIV-infected cells that have crossed the BBB as well as cell-free virus that has infected and traversed endothelial cells. HIV-infected mononuclear cells are most frequently encountered in perivascular macrophages, the antigen-presenting “dendritic” cell of the CNS [9]. Of possible importance in the pathogenesis of HIV-related neurological disease, perivascular macrophages turn over every two to three months, which may provide a continuous source of HIV-infected macrophages entering the brain. Tissue macrophages in brain parenchyma and microglia, the resident macrophage of the brain, also support HIV infection.

Astrocytes can support limited replication of HIV as well as express HIV-encoded proteins, including the virus regulatory protein, Nef [10]. Several investigators have suggested astrocytes may support HIV latency in the brain and could, when activated, upregulate HIV expression and replication [2, 8]. Replication of HIV in the astrocyte compartment may, therefore, lead to the appearance of new HIV variants within the brain. Neural infection by HIV is limited at best. Widespread infection of neurons by HIV does not occur, suggesting that neuronal cell death occurs by indirect pathways. Recently, investigators have found HIV viral DNA sequences encoding the viral regulatory genes *nef* and *gag* in hippocampal neurons [11]. Using laser capture techniques, genetic sequences encoding *nef* and *gag* were present in large numbers of neurons within the CA3 region of the hippocampus. Quantitative analysis suggests *nef* genetic sequences were more frequent than sequences encoding the Gag protein. If confirmed, these studies suggest that neuronal infection may be more frequent than realized and neurons may potentially express neurotoxic viral proteins that could be released from cells harboring viral genetic sequences. Oligodendrocytes do not support HIV infection.

The hallmark of HIV neuropathology is the presence of multinucleated giant cells (MNGCs) that are formed by fusion of HIV-1-infected macrophages with uninfected macrophages and possibly microglia [2]. The increase in activated macrophages and microglia are associated with increased production of cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL) 6, IL-1 β , and chemokines, including macrophage chemoattractant protein-1 (MCP-1) [12]. Upregulation of some chemokine receptors is also seen [13–15]. Evidence of neuronal apoptosis and oxidative stress is prominent in HIV-infected regions of the brain.

24.2.1.1 Chemokines in HIV-Related Neurological Diseases. Chemokines and chemokine receptors have an important role in HIV-related diseases. Chemokines belong to a superfamily of small (8–14-kDa) secreted proteins that were initially identified from their ability to regulate leukocyte trafficking during inflammatory responses [13]. Chemokines are highly conserved, pluripotent, and active in many different cell types. In addition to their chemotactic activity, chemokines have been implicated in modulation of cell adhesion, phagocytosis, cytokine secretion, cell activation, cell proliferation, apoptosis, angiogenesis, and viral pathogenesis. Chemokines and their receptors also have important roles in neural development, trophic support, and modulation of synaptic transmission. Expression of six human chemokine receptors have been described in adult brain: CCR2, CCR3, CCR5, CXCR2, CXCR3, and CXCR4.

Chemokine receptors function as coreceptors with CD4 for HIV-1 entry into target cells [13]. The cellular tropism of HIV-1 is determined by the interaction of HIV-1 gp120 envelope glycoprotein with a particular coreceptor. HIV strains isolated from brain use the CCR5 as a coreceptor. Macrophage-tropic (R5 or M-tropic) HIV-1 viruses use CCR5 as a coreceptor while T-cell-tropic (X4 or T-tropic) HIV-1 viruses use CXCR4 as their coreceptor. Dual-tropic viruses (R5 × 4) use both coreceptors. CCR2b, CCR3, CCR8, CXCR6, and CX3CR1 have been identified as “minor coreceptors” in tissue culture systems but not in vivo [15].

Upregulation of chemokines and chemokine receptors has been described in the brains of patients with HIV infection [13]. Neuronal and microglial expression of CXCR4 is found in regions of the limbic system and basal ganglia. CCR2 and CCR3 are upregulated in hippocampal neurons. CCR5 expression is not detected on neurons in the brains of AIDS patients. This suggests that CCR5 expression by neurons may be downregulated in HIV infection since CCR5 is expressed on neurons in normal brain. In patients with HIVE, chemokines and chemokine receptors are expressed most prominently on macrophages and microglia, especially in microglial nodules. CCR1, CCR3, CCR5, and CXCR4 are increased on macrophages and microglia along with expression of the respective chemokines MCP-1/CCL2, Macrophage-Inflammatory Protein-1 (MIP-1 α)/CCL3, and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES)/CCL5. Chemokine and chemokine receptor expression are most prominent in areas with histopathological signs of HIV infection. Chemokine levels in CSF correlate with severity of dementia and viral load in the brain as well as in plasma. MCP-1/CCL2, MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL5, IL-8/CXCL8, and fractalkine/CX3CL1 have all been found to be elevated in patients with HAD.

CXCR4 may be involved in gp120-mediated neurotoxicity either directly through activation of neuronal receptors by gp120 or indirectly by stimulation of astrocytes to release neurotoxic factors [16]. In primary neuronal cell cultures and neuronal cell lines, gp120 from T-tropic (X4) and dual-tropic (X4, R5) HIV strains induce apoptosis through CXCR4. In mixed neuronal/glia cell cultures, RANTES/CCL5 and MIP-1 β /CCL4 protect neurons from gp120-induced apoptosis by binding to CXCR4 receptors. The chemokine Stromal Cell-Derived Factor-1 (SDF-1 α)/CXCL12 does not prevent gp120-mediated neurotoxicity but can induce apoptosis in the absence of gp120. In vitro studies have shown binding of CCR5 by RANTES or MIP-1 α / β protects neurons from gp120-induced apoptosis. These findings are difficult to reconcile with the affinity for CCR5 shown by macrophage-tropic neurovirulent strains which are neurotoxic. Activated microglia and supernatant from activated microglia show enhanced neurotoxicity in CCR5-expressing neuroblastoma cells. It is possible that CCR5 acts as a death receptor in cells of neuronal lineage or binding of chemokines to CCR5 does not prevent cell death through other pathways.

Upregulation of the expression of chemokines and chemokine receptors is also seen in the simian immunodeficiency virus (SIV) model in macaques [17]. CCR3 and CCR5 are predominantly seen on perivascular macrophages and microglia although, in adult brains, CCR3 is also expressed on microvascular endothelial cells [15]. Increased expression of β -chemokines is found in the brains of monkeys with SIV encephalitis (SIVE) as they are in the brains of children and adults with HIV/AIDS (17to).

The findings of upregulation and downregulation of chemokine receptors and chemokines suggest they have a complex role in the pathogenesis of HIV-related neurological diseases. The effects of chemokines vary with cell type and experimental

conditions studied. In general, neurotoxic effects have not been attributed to β -chemokines in vivo while several in vitro studies have shown that β -chemokines have neuroprotective properties by protecting neurons against apoptosis induced by the HIV gp120 envelope glycoprotein [4]. α -Chemokines are present in the CSF and brains of HIV-infected patients.

In summary, changes in the expression of chemokines and their receptors are seen in HIV-infected brains. Upregulation of chemokines and chemokine receptors in HAD and HIVE support an important role for chemokines in HIV-related neurological disease. The functions of chemokines are pleiotropic with some having neuroprotective properties while others are likely to be involved in neurotoxic pathways. The functions of chemokines in HIV-related diseases depend on the type of chemokine and receptor, cell type, age of the patient, and HIV strain examined. A clearer understanding of the role of chemokines and their receptors awaits further study.

24.2.1.2 Mechanisms of Neurodegeneration in HIV-Related Diseases. The low level of HIV infection in the brains of HIV/AIDS patients, the absence of significant neuronal and astrocyte infection with HIV, the evidence of significant immune response in the brain despite low levels of HIV infection, and the disparity between the degree of HIV infection, and neurological symptoms suggest that the pathogenesis of HIV-related neurological diseases likely involves pathogenic pathways that do not require lytic infection of target cells. Current research suggests HIV infection of the brain leads to neural dysfunction through both the direct neurotoxic effects of viral proteins and indirect mechanisms utilizing immune mediators released by activated mononuclear cells in the brain. Direct and indirect neurotoxic pathways lead to cell death cascades similar to those that occur in AD and other neurodegenerative diseases. These findings suggest that HIV-related neurological diseases may best be considered degenerative diseases of the nervous system initiated by infection by HIV.

24.2.2 Neurotoxic HIV Proteins

Both HIV structural and regulatory proteins are neurotoxic either directly affecting neurons or indirectly by activation of astrocytes, macrophages, and microglial (Table 24.3).

24.2.2.1 Structural Glycoproteins gp120 and gp41. The HIV envelope protein gp160 is formed by an extracellular component, gp120, bound to the transmembrane anchor gp41. Both molecules have neurotoxic properties that may have a role in HIV-related neurological disease.

Glycoprotein gp120 is neurotoxic at extremely low concentration in vitro [18]. Picomolar concentrations (20 pM) of gp120 is toxic to cultured hippocampal neurons. The neurotoxicity of gp120 has also been demonstrated in vivo [19]. Intracerebral inoculation of mice with gp120 results in destruction of neurons around the injection site [20]. Transgenic mice expressing gp120 controlled by the glial fibrillary acidic protein (GFAP) promoter develop neuropathological changes including dendritic abnormalities in neurons, gliosis and age-related changes in long-term potentiation (associated with memory), open-field activity, and spatial reference memory [21]. The neuropathological findings seen in transgenic mice expressing gp120 are similar to those found in HIV-infected brains of patients dying with AIDS [21].

TABLE 24.3 Neurotoxicity of HIV-encoded Proteins

HIV Protein	Location	Toxic Concentration		Direct Toxicity	Indirect Toxicity
gp120	Membrane bound and extracellular	Picomolar		NMDA stimulation, EAA cytotoxicity, oxidative stress, apoptosis	↑NO, ↑tyrosine kinase, ↑cytokine release
gp41	Membrane bound and extracellular	Nanomolar		None known	Requires astrocytes, mitochondrial dysfunction, ↑ glutamate release
Tat	Extracellular (taken up by neurons and astrocytes)	Nanomolar		Non-NMDA and NMDA stimulation, Integrin binding, ↑intracellular calcium, EAA cytotoxicity, oxidative stress, apoptosis	Cytokine, chemokine, and metalloproteinase release, impaired astrocytes functions
Vpr	↑CSF, not extracellular	Micromolar		Creates Na ⁺ channels in membranes with inward cation current, caspase-8 activation	?
Nef	Intracellular	Nanomolar		? The <i>nef</i> gene sequences in neurons, activation of caspases, K ⁺ currents with membrane depolarization	Cytokine release, apoptosis of astrocytes, K ⁺ currents
Vpu	Intracellular	?		No known interactions with neurons; forms ion channels in COS cells	?
Rev	Intracellular	?		Interacts with neurons to alter cell membranes	?

Abbreviations: NMDA, N-methyl-D-aspartate; EAA, excitatory amino acid, COS, CV1 monkey cell line transformed by SV40 T antigen.

The mechanisms leading to gp120 neurotoxicity are complex and likely involve both direct and indirect effects. Glycoprotein 120 can directly alter neuronal functions through glutamate receptors. Both NMDA and kainic glutamate receptors bind gp120, resulting in increases in intracellular Ca^{2+} . Glycoprotein 120 binding to NMDA receptors results in increased intracellular calcium and excitotoxic cell damage [16, 22]. These effects are blocked by NMDA receptor antagonists memantine, {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate} (MK-801), and 2-amino-5-phosphonovaleric acid (APV), which protect against gp120-mediated toxicity in vitro [16, 22–25]. Interestingly, high concentrations of gp120 (100 nM) have been shown to be neuroprotective by acting as NMDA receptor antagonists [26].

Glycoprotein 120 may also exert toxic effects on neurons by indirect mechanisms. Glycoprotein 120 acts on astrocytes by stimulating an inducible form of nitric oxide synthase, inhibits β -adrenergic function, induces tyrosine kinase activity, modifies expression of adhesion molecules, and produces cytoskeletal changes [27]. Astrocytes normally protect neurons from excitotoxic damage by buffering glutamate. Glycoprotein 120 simulates Na^+/H^+ exchange, leading to alkalization and inhibition of Na^+ -dependent glutamate influx into astrocytes and preventing buffering of excess glutamate [28, 29]. Activation of monocytes and macrophages by gp120 promotes oxidative damage and increase the release of neurotoxic cytokines, including $\text{TNF-}\alpha$, $\text{IL-}\beta$, and prostaglandin E2 [30–33].

The transmembrane glycoprotein gp41 has also been implicated in neuronal toxicity. Glycoprotein 41 is elevated in the brains of HAD patients. In a postmortem study of HIV-associated dementia, the severity and rate of progression of HAD correlated with levels of gp41 along with inducible nitric oxide synthase (iNOS) and human alveolar macrophage 56 (HAM56), a marker of microglial/macrophage activation [34]. Although normally bound to plasma membranes, HIV gp41 was found to accumulate as an extracellular aggregate in the brains of patients with HAD [35]. In vitro, gp41 is neurotoxic in low nanomolar concentrations. Neurotoxicity requires the presence astrocytes, suggesting gp41 acts indirectly to impair neurons.

In a study using rat cortical slices, both gp41 and gp120 evoked very rapid release of glutamate, noradrenaline, and adenosine [14]. The release processes were too rapid to be accounted for by gene transcription and protein synthesis. The responses immediately returned to basal levels when the proteins were removed, supporting the specific effects of gp41 and gp120. In these experiments, gp41 was more effective than gp120 in evoking the release of glutamate and noradrenaline while both proteins were equally effective in adenosine release. The increase in glutamate from neurons exposed to gp41 is associated with impaired buffering of glutamate by astrocytes. Astrocytes treated with gp41 have deficits in both glutamate transport and release of glutathione [36–38]. Neuronal cell death related to gp41 involves multiple pathways, including activation of iNOS, NO formation, depletion of glutathione, and disruption of mitochondrial function [39].

24.2.2.2 Non-Structural HIV Proteins.

24.2.2.2.1 *Tat*. Tat is an important regulator of virus transcription. Tat-induced transactivation of the HIV-1 long terminal repeat (LTR) is essential for viral gene expression and virus replication. HIV-1-infected cells release Tat where the protein can be taken up by both astrocytes and neurons and rapidly translocated to the

nucleus [40]. Intraventricular injection of Tat is lethal to mice within hours of injection [41]. Cell loss occurs in selective subpopulations of neurons. Regions particularly susceptible to Tat neurotoxicity include the striatum, dentate gyrus, and CA3 region of the hippocampus [42, 43].

Tat is neurotoxic in the low nanomolar range [43–45]. Tat interacts with neurons through two mechanisms. It is thought that Tat acts directly through non-NMDA receptors whereas the stimulation of NMDA receptors by Tat is presumably a secondary effect of stimulation of non-NMDA receptors. Stimulation of glutamate receptors by Tat results in cellular excitotoxicity by either non-NMDA or NMDA receptors. The initial depolarizing response following exposure to Tat is inhibited by non-NMDA receptor antagonists whereas the resulting neurotoxicity measured hours later can be partially blocked by either non-NMDA or NMDA antagonists [45]. Depolarization of neurons by Tat does not show evidence of desensitization when Tat is repetitively applied to cultured neurons. The nondesensitizing actions of Tat may provide a mechanism for continuous cell depolarization over prolonged periods of time that could lead to excitatory amino acid cell cytotoxicity in the HIV-infected brain.

Tat also interacts with neurons by binding to cell surface integrins [46]. Tat binding to cell surface integrins may interfere with neuronal adhesion to the extracellular matrix that, in turn, alters cell signaling, migration, and other neuronal functions dependent on interactions between integrin receptors and the extracellular matrix. The upregulation of phosphorylation of the protein tyrosine focal adhesion kinase p125^{FAK} by Tat in neuronal cell cultures supports the notion that Tat acts through integrins since p125^{FAK} is involved in intracellular signaling via integrins [47, 48]. Interactions of Tat with cell surface integrins may also have a role in mediating neuronal cell aggregation by Tat [46]. The second exon of Tat contains the conserved Arg–Gly–Asp cell adhesion motif that binds integrins. The basic region of Tat present in the first exon also contributes to the cell adhesive properties of Tat. The cell adhesive properties of Tat may interfere with normal development and migration of neurons found in perinatal HIV-infected brains. Tat could conceivably alter remodeling after traumatic insults by inhibiting cell migration and attachment to extracellular matrix molecules. In addition, Tat has recently been shown to interfere with normal functions of neural progenitor cells that could alter neurogenesis in pediatric and, possibly adult, HIV-infected patients.

Tat may have neuroprotective properties at low concentrations. Neuronal cells transfected with tat complementary DNA (cDNA) or treated with low concentrations of exogenous Tat show increased resistance to apoptosis which can be blocked by anti-Tat antibodies [49, 50]. Upregulation of p125^{FAK} by Tat facilitates activation of phosphatidylinositol 3-kinase which is important in nerve growth factor prevention of apoptosis [47, 50–52].

Tat causes significant increases in the levels of intracellular calcium in neurons [52, 53]. Initially, the increase in calcium occurs through mobilization of the inositol triphosphate (IP³) sensitive pool followed by prolonged increase in the cytoplasmic calcium by influx of calcium from the extracellular fluid. This is followed by mitochondrial calcium uptake, generation of reactive oxygen species, and activation of caspases leading to apoptosis. Tat-induced neural cell death can be prevented by excitatory amino acid receptor antagonists, inhibition of nitric oxide synthase and caspases, antioxidants and agents that stabilize mitochondrial membrane permeability, and IP³ pools of intracellular calcium and inhibitors of glycogen synthetase kinase-3 β by lithium [53]. The importance of oxidative stress in Tat neurotoxicity is

supported by findings in rats receiving intrastriatal injection of Tat. Oxidative changes are found in proteins and lipids adjacent to the injection site. Tat appears to have an antioxidative effect in glial cells. However, once Tat is released from astrocytes, it causes oxidative stress and toxicity in neurons. Tat can induce expression of the neurotoxic chemokine SDF-1 in neurons which when released is toxic to adjacent neurons but not astrocytes.

In addition to its effects on neurons, Tat also alters glial and monocyte/macrophage functions. Tat promotes the release of neurotoxic agents, including matrix metalloproteinases (MMPs), IL-6, IL-8, RANTES, MCP-1, and TNF- α , from astrocytes and macrophages [54–58]. The release of MCP-1 and MMPs may increase the permeability of the BBB and promote transmigration of HIV-infected monocytes into the brain [58]. Taken together, these studies suggest Tat is an important mediator of the inflammatory response in the brain.

24.2.2.2.2 Vpr. Vpr regulates viral transcription and augments virion production [59]. Vpr is important for transport of the preintegration complex, nuclear localization, induction of cell cycle arrest in G2, and transactivation of the HIV-1 LTR and other cellular promoters. Thus far, the protein has not been shown to be present in the extracellular milieu in HIV-infected brains but is present in increased levels in the CSF and serum of HIV/AIDS patients with neurological disease [60]. In vitro studies have shown that extracellular Vpr is toxic to neurons in micromolar concentrations. Vpr causes a large inward cation current and caspase-8 activation. The N-terminal region of Vpr consisting of the first 40 amino acids is necessary and sufficient to form the sodium channel causing the inward cation current [61]. Similar effects are found when Vpr is expressed intracellularly in terminally differentiated neurons [62, 63]. Caspase-8 is an initiator caspase which, in turn, activates caspases 3, 6, and 7 leading to apoptotic cell death.

24.2.2.2.3 Nef. Nef is an HIV-1 regulatory protein that promotes both viral replication and immune evasion of HIV-1 [59]. Nef is required for proper budding of virions from HIV-infected cells. Abundant Nef expression is found in astrocytes in the brains of HIV-1-infected patients with HAD and HIVE [64]. Nef is also expressed by astrocytes in the macaque monkey model infected with SIV [65].

In vitro, Nef is lethal to neurons and astrocytes in nanomolar concentrations [66]. Transfection of astrocytes with a Nef-expressing vector results in astrocyte cell death [67]. Astrocytes expressing Nef release proinflammatory molecules, including IL-6, IL-1, and TNF- α , that are neurotoxic and can result in neuronal cell death. Nef can induce neuronal cell death by activation of caspases and free-radical production [66]. Nef may also be toxic through membrane depolarization [68]. Nef has amino acid sequence homologies to scorpion proteins [59]. Like scorpion proteins, Nef increases K^+ currents evoked after membrane depolarization.

Recent studies have shown HIV-1 *nef* DNA sequences are present in hippocampal neurons [11]. In these studies, both *nef* and *gag* cDNA sequences were found in CA region of the hippocampus using laser capture microdissection techniques. The highest number of *nef*-positive cells were found in the CA3 region with a rank order of CA3 > CA4 > CA1. The *nef* gene sequences were found in a greater percentage of neurons and in higher copy number than *gag*-coding sequences. The significance of these findings is as yet unknown. However, they do suggest that neurons may harbor and possibly express HIV proteins that have neurotoxic potential.

24.2.2.2.4 Vpu. Vpu is an integral membrane protein which is present in the intracellular milieu [69]. Vpu enhances virion release from HIV-1-infected cells. Like Tat and Nef, Vpu has not been found in the extracellular space in brains of patients with HAD and is not released by HIV-1-infected cells. No direct evidence of interactions between Vpu and neurons has been shown to occur. However, Vpu does form ion channels in planar lipid bilayers which are permeable to K^+ , Na^{2+} , and Cl^- [70]. Consistent with these findings are alterations in plasma membrane permeability of Vpu-transfected COS cells [71]. If Vpu forms ion channels in the membranes of neurons and/or glia, this would offer another example of HIV nonstructural proteins forming ion channels that could lead to membrane depolarization and altered cell functioning.

24.2.2.2.5 Rev. Rev is an essential regulator of viral transcription. The protein is involved in the regulation of splicing and transport of the viral RNA from the nucleus toward the cytoplasm and may be involved in the translation of viral transcripts [69]. Intraventricular injection of synthetic peptide spanning the basic region of Rev is neurotoxic and lethal to rodents [72]. Direct interactions of Rev with neurons have been demonstrated. The interaction of Rev with acidic phospholipids and the formation of α -helical conformations could possibly disrupt membrane topology that would be toxic and prove lethal [72].

In summary, both HIV structural and regulatory proteins have neurotoxic properties. HIV proteins exert their neurotoxic effects by a variety of pathways. Direct effects on glutamate receptors altering intracellular calcium can lead to excitatory cell toxicity. Several HIV proteins are capable of altering ion channels and cation movements across cell membranes. Oxidative stress follows changes in monocyte/macrophage and astrocyte functioning after exposure to HIV proteins. Cell death cascades are a common final pathway of cell death from the varied effects of HIV replication and expression of viral proteins.

24.3 CELL DEATH CASCADES IN HIV-RELATED NEUROLOGICAL DISEASE

The pathogenic pathways leading to cell death in HIV-related diseases are complex and involve direct and indirect effects of HIV on neurons, glial cells, macrophages, and lymphocytes [53]. In general, the mechanisms leading to neuronal cell dysfunction and death are similar to those seen in AD. In both HIV and AD, apoptosis, neurotransmitter-induced excitotoxicity, and inflammation lead to cell death. Whereas β -amyloid triggers cell death cascades in AD, HIV-1 proteins and proinflammatory molecules are the likely initiating factors in HAD and other HIV-related neurological diseases.

24.3.1 Apoptosis

The importance of programmed cell death in HIV-1-infected brains is supported by both in vivo and in vitro evidence of apoptosis. DNA fragmentation, caspase activation, and mitochondrial alterations seen in apoptosis are found in brains of patients dying with HAD [73]. The tumor suppressor protein p53 may mediate

apoptosis in HAD [74]. The p53 levels are increased in brains of HIV dementia patients. Cortical neurons lacking p53 are resistant to cell death induced by gp120 and Tat. The cell death associated with macrophage-trophic HIV isolates can be prevented by overexpression of Bcl-2, suggesting that the neurotoxic properties of these isolates are mediated by the mitochondrial apoptotic cascade [75]. The prostate apoptosis response-4 protein (Par-4) is believed to mediate apoptosis in neurons [76]. Par-4 levels are increased in neurons in the hippocampus of patients with HIVE and monkeys with SIVE. Par-4 is also increased in hippocampal neurons treated with Tat.

Neurotrophic factors protect neurons from apoptosis after exposure to Tat [77]. Brain-derived neurotrophic factor (BDNF), nerve growth factor, and activity-dependent neurotrophic factor protect cerebellar granular cells from the neurotoxic effects of Tat. The neuroprotective effects of neurotrophic factors are mediated by activation of nuclear factor NF- κ B and upregulation of Bcl-2. The cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) mediates activity-dependent neuronal survival by upregulating BDNF expression. Tat treatment of PC12 cells is followed by decreased levels of CREB expression and activity that preceded apoptosis [78]. These experiments suggest that neurotoxic proteins may inhibit pathways that promote neuronal survival as well as initiate apoptotic cascades.

The mechanisms of cell death in T cells and neurons appear similar including invoking cell death receptors, several cytokines, calcium dysregulation, increased mitochondrial membrane permeability, and caspase activation [53]. Tat and gp120 trigger apoptosis in different cell types. HIV-1-infected macrophages produce FLJ21908, a human proapoptotic factor produced by the chronically HIV-1 human macrophage hybridoma cell line, 43HIV, which is cytotoxic to CD4 and CD8 T cells, B cells, and neuroblastoma cells. FLJ21908 present in the brain and lymph nodes of patients with HAD. Increased levels of Vpr can kill cultured hippocampal neurons by forming ion-conducting pores in the plasma membrane, which in turn activates caspase-8 [63].

Culture media from HIV-1-infected T cells induce neuronal apoptosis only when virions are present. Supernatants from HIV-infected macrophages are toxic whether virions are present or not. Excitatory amino acids, NO, MMPs, and proinflammatory cytokines present in macrophage supernatants all may mediate the induction of apoptotic cascades. Drugs of abuse which are becoming an increasing comorbidity in HIV-infected diseases can also induce apoptotic pathways [79]. μ -Opioid receptors are widely expressed by astrocytes. Activation of μ -opioid receptors can induce apoptosis. Prolonged exposure of cultured striatal cells to Tat and morphine resulted in death of astrocytes by apoptosis.

24.3.2 Excitotoxicity Cell Death

Overactivation of glutamate receptors by gp120 and Tat illustrates the importance of excitotoxicity in cell death in HIV-1 infection. Glycoprotein 120 from HIV isolates associated with HAD can directly stimulate glutamate receptors and increase cell calcium concentrations [23]. Glycoprotein 120 promotes the release of arachidonic acid from glial cells which can then modify NMDA receptor kinetics increasing the mean open probability and channel open time [80]. The combined effects of increased glutamate concentrations in the extracellular space as a result of astrocytes dysfunction and the deregulation of the NMDA receptor results in increased calcium in

neurons and cellular dysfunction and death by disruptions of redox balance and sphingolipid metabolism [30–32]. Glutamate receptor antagonists inhibit gp120-induced calcium changes in neurons and astrocytes and protect neurons from gp120-induced cell death [30].

Tat can directly interact with neurons and has been shown to depolarize neural membranes independently of Na^+ flux [45]. Tat-evoked responses are similar to the glutamate agonist kainite. Unlike kainite, Tat does not desensitize non-NMDA receptors with repeated applications [81]. Desensitization of glutamate receptors inversely predicts toxicity, suggesting that the lack of desensitization with Tat is likely to lead to cell toxicity with repeated applications. In HIV-infected brains it is likely that non-NMDA receptors are exposed to Tat for long periods of time. Tat-induced neurotoxicity can be blocked with antagonists to excitatory aminoacid receptors. Tat potentiates both glutamate non-NMDA and NMDA excitatory aminoacid-triggered calcium influx and toxicity in cultured neurons [41, 45]. Concentrations of Tat and glutamate that are nontoxic induce cell death when combined. The toxicity of Tat is likely to be potentiated by proinflammatory cytokines and chemokines [82].

Tat-induced neuronal cell death is calcium dependent [53]. Tat evokes an initial burst of intracellular calcium from IP^3 -sensitive pools. Glutamate receptor-mediated increased calcium flux into cells follows mobilization of IP^3 -sensitive calcium release. Mitochondrial calcium uptake, disruption of redox balance, and caspase activation lead to neuronal cell death.

Calcium regulation in glial cells may also be altered in HIV-infected patients. CSF from patients with HAD disrupts cellular calcium homeostasis in cultured astrocytes [83]. CSF from HIV-infected patients without dementia does not alter calcium metabolism in astrocytes. Alterations in astrocyte calcium metabolism may explain the failure of nimodipine in a clinical trial since this L-type calcium channel antagonist does not protect astrocytes from perturbations of calcium flux.

24.3.3 Oxidative Stress

Brains from patients with HAD show evidence of oxidative stress [84, 85]. Membrane-associated oxidative stress correlates with disease pathogenesis and cognitive impairment, including increased levels of the cytotoxic lipid peroxidation product 4-hydroxynonenal (HNE) [86]. HNE covalently modifies proteins on cysteine, lysine, and histidine residues. Modified proteins impair function of membrane-motive adenosine triphosphatases (ATPases) and transporters for glucose and glutamate. Increased levels of HNE after exposure to gp120 and Tat may therefore have a prominent role in the neurotoxicity in HIV-infected brains. HNE is known to mediate oxidative stress-induced apoptosis in cultured neurons [87]. In vivo, HNE can damage neurons and cause cognitive dysfunction. Proinflammatory cytokines may potentate oxidative stress caused by gp120 and Tat. Tat-induced apoptosis of cultured human cortical neurons is potentated by $\text{TNF-}\alpha$ by increasing oxidative stress.

24.3.4 Effects of HIV on Neural Progenitor Cells

Mammalian brains have populations of progenitor cells that are capable of dividing and differentiating into neurons and glial cells [88]. In the hippocampus, neural progenitor cells are present in the dentate gyrus where they may participate in

hippocampal-dependent memory [89, 90]. Neurogenesis by adult progenitor cells is reduced by aging and toxic stimuli, including alcohol, methamphetamine, opiates, β -amyloid, encephalitis and inflammation, and stress [91–93]. Neural progenitor cells express CXCR4 and CCR5 chemokine receptors, suggesting these cells are likely to be susceptible to HIV infection [93, 94].

The number of proliferating neural progenitor cells are reduced in the hippocampus of HIV-1-infected patients [95]. HIV-1 can infect neural progenitor cells in vitro [53]. The effects of HIV infection in neural progenitor cells are unclear, but preliminary studies suggest impaired ability to divide and differentiate [95].

HIV infection of neural progenitors along with the ability of Tat to bind to neuronal membrane integrins may provide insight into the effects of HIV on the developing brain. Further study of the effects of HIV on neural progenitor cells and neural development are likely to provide an increased understanding of the effects of HIV in pediatric patients as well as possibly adults with cognitive impairment.

24.4 ALTERATIONS IN LIPID METABOLISM IN HIV

Alterations of lipid metabolism appear to have an important role in HIV-related neurological diseases. Cholesterol, ceramide, and sphingomyelin levels are increased in the brains and CSF of HAD patients [86]. Membrane lipid peroxidation and increased ceramide production are likely to be central events in the death of neurons since gp120 and Tat increase cellular levels of HNE and ceramide. The ceramide precursor palmitoyl-CoA increases the sensitivity of neurons to gp120 and Tat neurotoxicity while inhibitors of ceramide production reduce gp120 and Tat neurotoxicity [86]. HIV infection may promote lipid imbalance in neural cells.

Dysregulation of lipid and sterol metabolism in HAD is associated with the E4 genotype that is also associated with increased risk for AD [96]. Levels of sphingomyelin, ceramide, and cholesterol are significantly increased in the medial frontal cortex, parietal cortex, and cerebellum in HAD patients with E3/4 and E4/4 genotypes compared to those with E3/3 genotype [87].

Ceramide production is upregulated in neurons exposed to gp120 and Tat [97]. Both gp120 and Tat induce sphingomyelinase activity by invoking oxidative stress by CXCR4 activation. Glycoprotein 120 has a galactosylceramide binding domain which has been associated with the regulation of membrane trafficking from the golgi to lipid rafts [98].

Lipid rafts are membrane microdomains that may have an important role in the pathogenesis of HAD [86]. Lipid rafts are regions of the plasma membrane that contain high levels of cholesterol and sphingomyelin. Receptors for cytokines, chemokines, and growth factors are concentrated in lipid rafts. These membrane structures are believed to be the portals through which HIV-1 and other viruses enter cells [86, 92, 99]. It remains to be shown whether gp120, Tat, and other HIV-encoded neurotoxins alter the structure or function of lipid rafts.

24.5 NIGROSTRIATAL SYSTEM IN HIV/AIDS

The nigrostriatal system has a central role in HIV-related neurological disease (Table 24.4). HIV-infected individuals exhibit a wide range of symptoms and signs

TABLE 24.4 The Nigrostriatal System in HIV/AIDS

Clinical disorders have characteristics of basal ganglia disorders.
Dopamine levels depleted early in the clinical course.
Dopaminergic neurons appear to be specifically targeted by HIV-related neurotoxic pathways.
Dopamine acts synergistically with HIV Tat and possibly other viral proteins in neurotoxic pathways.
Dopamine and dopaminergic drugs accelerate SIVE in monkeys and possibly HIVE in humans.
Dopamine upregulates HIV expression in peripheral blood mononuclear cells.

directly attributable to dysfunction of dopaminergic systems. Basal ganglia involvement by HIV occurs early in the disease as evidenced by slowed cognition and motor reaction times even in asymptomatic HIV-infected patients. Impaired psychomotor speed is often the first manifestation of HAD [100]. With disease progression, patients develop a subcortical dementia clinically indistinguishable from that encountered in patients with PD, Huntington's disease, and other disorders of the basal ganglia. Associated with the subcortical dementia are extrapyramidal motor signs, including bradykinesia, postural instability, gait abnormalities, hypometric facies, slowed reaction times, and decreased rapid alternating movements.

Several lines of evidence support involvement of the dopaminergic system in HIV/AIDS. As noted above, patients who develop HAD had many features reminiscent of subcortical dementias seen in degenerative diseases of the basal ganglia. A smaller number of patients develop a parkinsonian syndrome indistinguishable from idiopathic PD [3]. The predominant neuropathological features of HIV/AIDS are found in the putamen and caudate nuclei of the basal ganglia [101]. HIV infection is most frequently found in the basal ganglia. The HIV envelope glycoprotein gp41 and the core protein p24 are found predominantly in the dopamine-rich regions of the basal ganglia [12, 102]. Neuronal degeneration is present in the substantia nigra in brains of patients dying in the late stages of HIV/AIDS [103]. Using cell-counting techniques, significant neuronal cell loss has been noted in the pars compacta of the substantia nigra [104]. The remaining cell bodies are more heavily pigmented and shrunken in size. Gadolinium enhancement in the basal ganglia and reduced basal ganglia volume on magnetic resonance imaging (MRI) have been reported in HIV/AIDS patients with dementia but not those without cognitive changes [3, 105]. Altered striatal glucose metabolism is found using positron emission tomography (PET) scanning. Hypermetabolic changes are seen in the early stages of HIV disease while hypometabolic alterations are encountered during the late stages of HIV infection [106]. Finally, HIV-infected individuals are extremely sensitive to dopaminergic blocking agents and dopamine agonists [3]. Even extremely low doses of these medications can result in profound and permanent parkinsonism.

Alterations in dopamine metabolism have been described in HIV-infected patients. Dopamine concentrations are reduced in brain structures associated with the nigrostriatal dopaminergic pathway. CSF dopamine (DA) and homovanillic acid levels are reduced in patients with HIV-1 infection, the lowest levels being found in those with HAD [3]. Recently, abnormalities in dopaminergic transporters have been demonstrated in patients with HAD [107]. Using PET to assess availability of dopamine transporters ($[^{11}\text{C}]$ cocaine) (DATs) and dopamine D_2 receptors

([^{11}C]raclopride), HIV patients with HAD were found to have significantly lower DAT availability in the putamen and ventral striatum when compared to normal controls and HIV-infected patients without dementia. A higher plasma HIV viral load in HAD patients correlated with lower DAT in the caudate and putamen. Dopamine D_2 receptor availability was either mildly impaired or normal in HAD patients. The greater DAT decrease in the putamen than caudate as seen in HIV-1-infected patients with dementia parallels those changes observed in PD. The inverse relationship between HIV plasma viral load and DAT availability supports a direct relationship between HIV infection and reduced DA transport.

The HIV proteins gp120 and Tat are neurotoxic in selected populations of neurons [108]. Although these subpopulations of neurons are not completely defined, several studies have shown that dopaminergic neurons are particularly susceptible to the neurotoxic effects of HIV proteins. Injection of Tat into the lateral ventricles of rats results in apoptosis of striatal neurons [44]. Nuclear magnetic resonance spectroscopy shows loss of the *N*-acetyl-aspartate peak suggesting neuronal cell loss [109]. Pathological studies have shown loss of nigrostriatal fibers following injection of Tat peptides into the striatum. The excitatory amino acid receptor antagonist MK801 and blockers of NOS protected against loss of these cells [43]. Treatment of mesencephalic neurons with gp120 blocked DA uptake by neurons and resulted in loss of dopaminergic neuronal processes without affecting the number of cells [110]. Tat inhibits tyrosine hydroxylase activity as well as synthesis and release of DA from catecholaminergic cell lines [111]. Taken together, these studies suggest Tat and gp120 may alter DA metabolism and availability by destruction of dopaminergic neurons through excitatory amino acid cytotoxicity and cell death cascades and by altering DA synthesis and DA uptake.

In the SIV-infected macaque, DA levels are reduced in the striatum within the first two months of infection. Reduction in DA content is associated with an increase in the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) late in the disease [112]. The low levels of DA seen early in the disease are likely to be secondary to increased DA turnover as suggested by increased CSF concentrations of DOPAC in asymptomatic monkeys. In addition, cAMP and CREB, two factors involved in DA signaling pathways, are also altered in SIV-infected macaques within the first 4–19 weeks after suggesting that DA signaling may be altered early in infection [113]. The findings in SIV-infected monkeys suggest that alterations in DA metabolism and signaling may also occur early in HIV-infected patients.

24.5.1 Response to DA Mediators

Several medications commonly administered to HIV-infected patients directly modulate dopaminergic neurotransmission [112]. HIV/AIDS patients, even when treated with mild DA-blocking drugs, such as prochlorperazine, perphenazine, trifluoperazine, low-dose haloperidol, thiothixene, chlorpromazine, or metoclopramide, may develop severe and permanent parkinsonism [114–117]. In one study, the likelihood of developing parkinsonism was two- to fourfold greater in patients with AIDS when controlled for mean drug dose and body weight [118]. Parkinsonism developed in 50% of AIDS patients who received less than 4 mg/kg of chlorpromazine equivalents per day and 78% of those who received more than 4 mg/kg of chlorpromazine equivalents per day.

L-Dopa has been shown to reverse some parkinsonian symptoms in a subset of HIV-infected patients [116, 119]. DA receptor agonists consistently improve motor dysfunction in pediatric patients with HIV and signs of parkinsonism [119]. However, the response to DA receptor agonists in adult HIV-infected patients with parkinsonism has been variable [119a]. Selegiline, a monoamine oxidase-B inhibitor, improves cognitive deficits in HIV-infected patients and SIV-infected monkeys [120].

Psychostimulants are often given to HIV patients to treat depression and cognitive slowing. These drugs modulate dopaminergic neurotransmission through inhibition of DA reuptake and/or release of DA through calcium-independent pathways [121, 121a]. Psychostimulants increase cognitive performance and reduce fatigue and depression, common complaints in HIV-infected patients [123–125].

Concerns have been raised of the possibility that dopaminergic agents may accelerate HIV-related disease. DA is clearly toxic to neurons and may act synergistically with HIV-encoded proteins and other agents, including drugs of abuse (see below). Several instances of acceleration of dementia in patients with HAD who were treated with dopaminergic agents have been reported. For instance, Nath and co-workers reported a patient with HAD whose dementia rapidly worsened after treatment with psychostimulants [126].

Both L-dopa and selegiline accelerate SIV infection in macaque monkeys [127, 128]. Selegiline and L-dopa increase DA release and availability in macaque monkeys. The incidence and severity of SIVE was significantly increased in drug-treated animals. Neuropathological changes of SIV infection appeared earlier in monkeys receiving dopaminergic agents when compared to untreated controls. Drug-treated SIV-infected monkeys had increased SIV viral load in the brain, increases in the severity of infection-related neuropathological changes, and ultrastructural alterations in dendrites of neurons in dopaminergic regions of the brain within 8 and 20 weeks after infection. A spongiform polioencephalopathy restricted to dopaminergic areas of the brain was frequently encountered. This is a unique neuropathological change that is not found in SIV-infected macaques that have not been treated with dopaminergic agents. L-Dopa and selegiline both increased the expression of TNF- α mRNA levels in microglia in SIV-infected monkeys treated with L-dopa or selegiline, suggesting TNF- α may contribute to the synergism between SIV and dopaminergic agents through its neurotoxic effects [129]. The rapid appearance of neuropathological changes and viral load in SIV-infected monkeys treated with dopaminergic agents suggests that synergistic interactions between SIV and dopaminergic agents can provoke the early appearance of SIVE with enhanced levels of microglia-derived SIV and TNF- α in the absence of immunodeficiency [128]. Similar synergism between HIV and dopaminergic agents may explain, at least in part, the acceleration of HIV-related neurological changes in HIV-infected patients taking dopaminergic agents, including drugs of abuse (see below).

Dopaminergic agents may also alter HIV expression in peripheral immune cells. Dopaminergic agents act synergistically on lymphoid cells to alter immune responses. Lymphocytes produce, transport, and bind DA present in the plasma. Circulating monocytes, T lymphocytes, and B lymphocytes have DA receptors and mononuclear cells may synthesize DA. DA upregulates HIV expression in a dose-dependent manner in T lymphoblasts chronically infected with HIV-1 [130]. DA-induced activation of HIV infection was attenuated by glutathione and *N*-acetylcysteine,

suggesting DA acts by inducing oxidative stress through glutamate-dependent mechanisms. Support for this mechanism is found in the altered cellular redox states found in DA treated T lymphoblasts. HIV activation appears to be tightly linked to intracellular oxidant/antioxidant levels that are perturbed by DA. DA effects on HIV transcription are mediated through the NF κ B element within the LTR of HIV [131]. Dopaminergic effects on HIV replication in peripheral lymphoid cells may be augmented by proinflammatory cytokines. For instance, HIV replication is increased 15-fold in the presence of TNF- α .

24.6 OTHER NEUROTRANSMITTER SYSTEMS IN HIV-ASSOCIATED DEMENTIA

24.6.1 Excitatory Amino Acid Neurotransmitters in HAD

The effects of HIV gp120, gp41, and Tat on glutamate-mediated neurotoxicity have been reviewed above. Neuronal apoptosis following exposure to the viral proteins gp120 and Tat can be blocked by receptor antagonists for which excitatory neurotransmitters such as glutamate act as a substrate [23, 108]. Plasma glutamate levels are significantly elevated in HIV-infected patients, including those who are asymptomatic [132]. CSF glutamate levels are increased in patients with HIV/AIDS with and without HAD [133]. These findings are consistent with decreased astrocyte uptake of glutamate from the synaptic cleft, elevated plasma glutamate, or both. NMDA receptor density is consistently reduced in postmortem studies of patients with HAD, suggesting alterations of receptor synthesis following chronic overstimulation. Experiments using the SIV-infected macaque monkey model suggest alterations in glutamate metabolism occur early during the course of the disease. CSF levels of glutamate, but not aspartate, begin to increase 11 weeks after SIV infection [134]. The increase in glutamate appears to originate in microglia and correlates with high levels of viral antigen.

Other excitatory amino acid receptor agonists may possibly be involved in HAD. The most extensively studied of these is quinolinic acid. Quinolinic acid, an endogenous metabolite of L-tryptophan, is increased early and remains elevated in the CSF of HIV-infected patients [135]. Activated macrophages, both HIV infected and uninfected, produce and secrete quinolinic acid and are the likely source of quinolinic acid in HIV-infected brains and CSF [136]. Elevated CSF levels of quinolinic acid correlate with dementia in the late stages of HIV/AIDS and in SIV-infected monkeys with neurological symptoms [17, 137].

24.6.2 Cholinergic System in HAD

Essentially nothing is known about the cholinergic system in patients infected with HIV. Neuropsychological deficits are encountered frequently in HIV-infected patients before the development of HIV/AIDS [5]. Early signs of cognitive decline in HIV-infected patients may be dominated by impaired attentional deficits and reductions in perceptual and psychomotor speed [138]. The cortical cholinergic system has an essential role in attentional functions and regulation of processing capacity, suggesting this system may have a role in HIV-related abnormalities in

these cognitive domains [139]. Unfortunately, neuropathological studies of the brains of patients with HIV/AIDS have not addressed changes in the cholinergic system. However, the cholinergic system has been studied in SIV-infected monkeys. The activity of choline acetyltransferase (ChAT) is significantly reduced in the SIV-infected macaque [140]. Reduced ChAT activity was most prominent in the putamen and hippocampus in asymptomatic monkeys. These changes did not correlate with brain SIV load, neuropathological changes, or alterations in systemic immunity. ChAT activity could be completely restored by selegiline at doses that possess dopaminergic activity [141]. The findings in the SIV-infected monkey and the cognitive changes seen early in HIV-infected individuals suggest that further attention to alterations in cholinergic transmission in HIV/AIDS may provide valuable insight into the pathogenesis of perceptual alterations and memory deficits in HIV-infected patients.

24.7 DRUGS OF ABUSE IN HIV/AIDS

The pharmacological effects of drugs of abuse on HIV-related neurological disease is becoming of increasing importance. The role of drugs of abuse in facilitating risky behavior that leads to HIV infection was recognized early in the HIV/AIDS pandemic. During the ensuing years, drugs of abuse have been increasingly recognized as a prominent risk factor for HIV/AIDS. The most recent evidence suggests the HIV epidemic is, in part, being driven by drug abuse [142]. The fastest growing populations with HIV infection in the United States and Western Europe acquire HIV through illicit drug use. Drugs of abuse appear to accelerate HIV infection and increase the severity of HIV-related disease as illustrated by the increased prevalence of HIV among drug abusers [143]. The pharmacological effects of drugs of abuse impact the same neuropharmacological systems altered by HIV infection. Drugs of abuse can increase HIV viral load by upregulating HIV replication in mononuclear cells.

Studies assessing the frequency of drug abuse in patients with HAD have produced variable results. Grassi found a negative influence of drug abuse on cognitive function among Italian HIV-infected individuals [144]. The MAC study also failed to find a difference in the incidence of HAD in HIV-infected drug users [145]. A subsequent study reported that a history of injection drug use and psychomotor slowing at presentation heralded more rapid progression of neurological disease that was associated with more abundant macrophage activation in the brain [146]. A cohort study in Scotland found that 56% of the brains of HIV-infected drug users had features of HIV, including the presence of p24 antigen and multinucleated giant cells, compared to only 17% of homosexual HIV-infected men without a history of drug abuse having features of HIV [143].

The commonly abused drugs, including amphetamines, cocaine, and opiates, have dopaminergic activation properties which could accelerate the loss of dopaminergic neurons in an already compromised dopaminergic system in HIV-infected patients (Table 24.5). Supporting this contention is the finding of more severe neuronal cell loss and shrunken neuronal cells in the substantia nigra in HIV-infected drug users when compared to HIV-infected patients without a history of drug abuse [104].

TABLE 24.5 Drugs of Abuse and Synergism with HIV Proteins

Drug	Mode of Toxicity	HIV protein synergism
Methamphetamine	Oxidative stress, mitochondrial damage	Tat: increased oxidative stress/apoptosis
Opioid	Decreased dopamine levels, apoptosis	Tat: increased oxidative stress/apoptosis
Alcohol	Sensitize glutamate receptors	Glycoprotein 120, Tat increased EEA toxicity

24.7.1 HIV and Methamphetamine/Cocaine

Methamphetamine and cocaine act synergistically with HIV Tat and gp120 in neurotoxicity studies of dopaminergic systems. Neuronal cell lines exposed to dopamine, cocaine, or morphine along with supernatants from HIV-infected cells show significant cell death and oxidative stress [147]. Acute exposure of human neuronal cell cultures to methamphetamine and cocaine in the presence of gp120 or Tat results in oxidative stress and cell death in a subpopulation of neurons [148]. The mechanism of synergy in these studies appears to be, at least in part, through alterations in mitochondrial membrane potentials leading to oxidative stress. 17 β -Estradiol prevents the synergistic toxicities of methamphetamine and cocaine with dopamine in these studies [149, 150].

Oxidative stress is a possible mechanism for the synergism between cocaine and HIV proteins. Mitochondria are critical cellular targets for cocaine toxicity as evidenced by decreased mitochondrial respiration and increased synthesis of reactive oxygen species in animals exposed to cocaine. HIV Tat also produces oxidative stress in primary neuronal cell cultures with alterations in mitochondrial membrane potentials similar to those seen with cocaine. Oxidative changes in cellular proteins occur rapidly after injection of Tat into the rat striatum. Since both cocaine and Tat produce oxidative stress by targeting mitochondria, cocaine and HIV could have synergistic interactions in producing mitochondrial dysfunction, oxidative stress, and cell death.

Clinical and experimental evidence suggests methamphetamine and HIV act synergistically to alter neural functions. Nath reported the acceleration of HAD in a HIV-infected patient using methamphetamine [126]. Magnetic resonance spectroscopy studies in HIV-infected patients with a history of chronic methamphetamine use suggest an additive effect of methamphetamine with HIV [150]. The neuronal marker *N*-acetylaspartate was reduced most prominently in the basal ganglia of HIV-infected patients who were chronic methamphetamine users compared to HIV-infected patients without a history of drug abuse. The glial cell marker *myo*-inositol was significantly increased in the frontal lobes of these same patients. The authors suggest the reduction in *N*-acetylaspartate in the basal ganglia reflected neuronal cell loss while the increase in frontal lobe *myo*-inositol could be explained by astrocytosis following upregulation of HIV replication by methamphetamine.

Methamphetamine and HIV act synergistically in cell culture studies of neurotoxicity. Treatment of human neuronal cell cultures with methamphetamine or cocaine along with gp120 or Tat results in neuronal cell death that is prevented by treatment with 17 β -estradiol [150]. These neurotoxic effects were associated with

mitochondrial membrane potential changes and oxidative stress. Only a subset of cultured neurons was affected. Several studies have suggested that the subset of neurons affected in these studies is dopaminergic neurons.

HIV-1 Tat acts synergistically with methamphetamine to deplete striatal dopamine levels in an *in vivo* rat model [149]. HIV-1 Tat potentiates methamphetamine-induced decreases in overflow of dopamine in the rat striatum [151]. Injection of Tat into the rat striatum followed by intraperitoneal methamphetamine results in a 70–78% reduction in striatal dopamine overflow and content compared to 20% reduction in amphetamine-evoked overflow of dopamine and 16% decrease in dopamine content in rats treated with Tat alone. The interactions are specific for Tat and methamphetamine since neither heat-inactivated bovine serum nor HIV-1 gp120 was shown to synergize with methamphetamine. The synergism was also neurotransmitter specific since methamphetamine and Tat treatment did not alter serotonin levels. DOPAC levels are reduced in rats treated with Tat and methamphetamine. DOPAC levels reflect intraneural dopamine catabolism in dopamine synapses. The reduced DOPAC levels in rats treated with Tat and methamphetamine suggest either destruction of dopamine synapses has occurred or dopamine catabolism is shifted to another compartment. Tat alone increases the level of homovanilic acid (HVA), a product of extraneural dopamine metabolism. HVA levels were unaffected in rats treated with Tat and methamphetamine. These findings suggest that Tat and methamphetamine may have different effects on the degradation pathways for dopamine metabolism and/or destruction of dopamine terminals by the combination of Tat and methamphetamine prevents synthesis of DOPAC. Loss of dopamine terminals may reflect repeated exposure to methamphetamine. Administration of methamphetamine to experimental animals, and possibly humans, over prolonged periods of time results in destruction of dopamine terminals and reduced striatal dopamine levels [152].

Oxidative stress appears to be the primary mechanism leading to loss of dopaminergic neurons and dopamine in animals treated with Tat and methamphetamine. Mitochondrial membrane potentials are reduced in neurons exposed to HIV-1 Tat and methamphetamine [148, 149]. The combined toxicity of Tat and methamphetamines is blocked by antioxidants and 17 β -estradiol. These studies suggest that, even though mitochondria may be a source of reactive oxygen species in other circumstances, the blockade of oxidative stress by antioxidants in Tat and methamphetamine treated neurons suggest oxidative stress precipitates rather than results from mitochondrial dysfunction when Tat and methamphetamine are present together. This finding is not unprecedented since several mitochondrial complexes can be inactivated by oxygen reactive species [153, 154].

Methamphetamine also alters HIV expression outside the nervous system. Methamphetamine stimulates HIV replication in human peripheral blood mononuclear cells by increasing production of TNF- α [155]. These findings suggest that methamphetamine may increase viral loads by altering cytokine synthesis and release. The effects of methamphetamine on immune cell functions need to be more fully addressed. Increased HIV plasma loads have been found in patients who are using methamphetamines [156]. While upregulation of HIV replication would appear to be the most likely explanation for these findings, increased HIV plasma loads were only seen in patients taking HAART therapy. These paradoxical findings are most likely explained by poor compliance with HAART therapy by patients

abusing methamphetamine. Poor compliance in the timing and dose of HAART therapy could allow for the escape of viral mutants that replicate despite suppressive therapy. Whether such changes in HIV replication occur in the CNS has not been studied. The acceleration of HAD in patients taking methamphetamine suggests this may be a possibility that deserves further study.

24.7.2 Opioid Drugs

Opioids are typically proapoptotic when they cause cell death. Such μ receptor drugs as morphine and fentanyl induce toxicity in cerebellar Purkinje cell cultures and in the limbic system of rats when given in high doses [157, 158]. Fentanyl exacerbates the effects of ischemia-induced damage to the basal ganglia [158]. When combined with proapoptotic agents, opioids can exacerbate cell death [159–161]. For instance, μ agonists enhance staurosporine- and wortmannin-induced apoptosis in embryonic chick neurons or neuronal cell lines [162, 163].

Opioids may alter the susceptibility of dopaminergic neurons to viral damage by altering dopamine turnover. Endogenous opioids and opiate drugs interact with dopaminergic neurons through several pathways [164–166]. Opioids decrease dopamine levels by reducing the activity of inhibitory interneurons that synapse with dopaminergic neurons in the ventral tegmentum. Opioids also influence dopamine cellular responses directly. Opioid receptor agonists increase D_2 but not D_1 dopamine receptor binding sites in rat striatum [167, 168]. Repeated intermittent morphine exposure increases D_2 receptor-induced adenyl cyclase activity in the rat striatum.

Disruption of dopaminergic function with the development of tolerance and dependence or during opioid withdrawal may be of more significance when considering the effects of opioid drugs on HIV/AIDS [164]. Chronic opioid drug exposure is accompanied by disruption of second-messenger cascades, altered patterns of gene activation, and increased oxidative stress [164–166]. These changes, especially those leading to oxidative stress, may increase neuronal susceptibility to HIV neurotoxic pathways.

Opioids and HIV Tat protein act synergistically to produce neuronal cell death. Human and mouse neurons undergo programmed cell death when exposed to Tat and morphine. Opioid antagonists prevent apoptosis in cells exposed to Tat and morphine [169]. The synergistic effects of morphine and HIV Tat are mediated by mitochondrial toxicity acting through Akt kinase, PI^3 kinase, and caspases 1, 3, and 7 [142]. As seen in dopamine-induced neurotoxicity, opioids are toxic to only a subset of neurons. Neuronal susceptibility to apoptosis following exposure to HIV-encoded proteins and opioids may depend, at least in part, on the distribution of opioid receptors in the brain. Subpopulations of striatal astrocytes and microglia express opioid receptors and may mediate the toxic effects of morphine and Tat on neurons [170, 171]. Finally, the administration of exogenous opioids may alter endogenous opioid peptide levels that could also participate in neurotoxic pathways.

Opiates may alter HIV expression outside the nervous system. Opioid drugs and HIV proteins act synergistically to destabilize immunity by altering monocyte and lymphocyte functions. The “opiate cofactor hypothesis” has been proposed as a mechanism in the pathogenesis of HIV/AIDS [172]. The hypothesis is based on the findings that opioids promote HIV replication in immune cells and suppress immune

functions. Subpopulations of leukocytes express μ -, δ -, and κ -opioid receptors. Opioid drugs can modulate neuroimmune functions through direct and indirect actions that involve peripheral and central neural mechanisms. Opiates may have contradictory effects on immune functions depending on the receptor type involved. For instance, μ receptor stimulation is followed by increased HIV expression in monocytes while κ receptor activation leads to inhibition of HIV expression in monocytes and lymphocytes [155, 171, 173]. Opiates also increase the expression of chemokine receptors that serve as coreceptors for HIV infection in susceptible cells thus possibly increasing the number of HIV-susceptible immune cells [174].

24.7.3 Alcohol

Alcohol, like other drugs of abuse, is likely a significant risk factor for HIV infection. Individuals who abuse alcohol often engage in high-risk sexual behavior and have significant compromises of immune function from both the direct effects of alcohol and nutritional deficiencies [175–177]. Alcohol abuse can therefore alter both immune system function and nutritional status, which could potentially increase the risk for development of HIV/AIDS-related diseases such as HAD [178–180].

Chronic alcohol intake stimulates the synthesis of reactive oxygen species, inhibits neuronal growth factor expression, and reduces cerebral glucose utilization [181–183]. These changes from chronic alcohol exposure could render neurons and glial cells more susceptible to the neurotoxic effects of HIV-related pathogenic pathways.

Chronic alcoholism alters neuronal function and sensitizes glutamate neurotransmitter receptors, making them more susceptible to damage by HIV-1 neurotoxins. The neurotoxic effects of Tat and gp120 have been shown to be, at least in part, mediated by NMDA receptor overactivity, leading to calcium-mediated excitatory cell death [142, 184, 185, 188]. Adaptive changes in NMDA receptors during long-term chronic alcohol abuse have been well documented. Chronic ethanol exposure in animals and primary neuronal cell cultures results in compensatory increases in the density and sensitivity of NMDA-type glutamate receptors in cortical and hippocampal regions [186, 187]. NMDA receptor-mediated elevations in intracellular Ca^{2+} during alcohol withdrawal can result in neuronal cell death [189]. The NMDA receptor-mediated cell death in chronic alcoholism is blocked by NMDA receptor antagonists [183, 186, 188–190]. Alterations in NMDA activity seen with chronic alcohol abuse could potentiate the neurotoxic effects of Tat and/or gp120 acting through excitatory amino acid cell death cascades [108]. The oxidative stress seen in chronic alcohol exposure may also enhance that seen as a result of HIV-encoded proteins. Thus, chronic alcohol exposure and alcohol withdrawal may act synergistically with HIV by increasing cell death through either excitatory amino acid or oxidative cell death cascades.

24.8 NEURODEGENERATIVE DISEASES IN HIV/AIDS

HAART therapy has reduced mortality in patients infected with HIV [2]. While the incidence of HAD and other neurological complications of HIV has declined, the prevalence of HAD has increased as the HIV-infected population lives longer. The increased age of HIV-infected patients using HAART is likely to provide new challenges as neurodegenerative diseases of later life begin to complicate the course of

HIV/AIDS. Neuronal cell damage from HIV is likely to result in increased susceptibility to neurodegenerative diseases that may appear earlier than expected because of HIV-related compromise of neurotransmitter systems and other neural functions.

Cognitive decline appears to be more frequent and severe in older HIV-infected patients. In a community-based, sentinel survey of HIV-infected patients with cognitive impairment, the prevalence of cognitive disorders among HIV-infected individuals was significantly higher in those over 50 years of age compared to those less than 50 years old [184]. In the over-50 age group, dementia was the most common cognitive disorder seen, whereas milder cognitive impairment was more common in the under-50-year-old cohort. In these patients, alcohol was a significant risk factor for dementia whereas greater education was a protective factor. HIV viral load on entry into the study was significantly higher among those who developed cognitive impairment over the year of the study.

The demographic changes during the HAART era suggest patients may be at increased risk for developing AD and possibly other neurodegenerative disorders. Although speculative, there are several reasons for this concern. Increasing age, high serum lipids, axonal injury, and effects of Tat and quinolinic acid on the brain may lower the threshold for development of AD. The increased mean age in a prevalence survey of neuropsychological abnormalities in an outpatient tertiary clinic was 49 ± 8.8 years compared to 38.5 ± 7 years [6]. The number of patients with elevated cholesterol and triglyceride levels is increasing as a result of age, HIV disease itself, and HAART therapy, especially protease inhibitor drugs [13]. Elevated cholesterol levels have been consistently shown to be a risk factor for AD. Patients with axonal injury are also at increased risk of developing AD [191]. Axonal injury is a consistent finding in the brains of HIV-infected individuals, even early in the course of the disease. Deposition of β -amyloid in the brains of HIV-infected patients and alterations of β -amyloid metabolism in HIV infection may increase the risk of AD in these patients.

While the above is speculative, studies have shown deposition of β -amyloid in the brains of HIV/AIDS patients. A strong association between HIV and β -amyloid precursor protein (APP) deposition has been documented in several studies. The distribution of HIV p24 correlates with the distribution of APP aggregates [192]. APP aggregates often take the form of intra-axonal inclusions indicating that axonal injury has occurred. APP may also be found in extracellular aggregates in close proximity to HIV antigens. Similar APP depositions have also been found in SIV-infected macaques [193].

Esiri first described the presence of APP depositions in the brains of AIDS patients [194]. A more comprehensive study found perivascular APP plaques that stained with 4G8, an antibody that precipitates A β , suggesting the deposits could be primarily vascular in origin. Green found a statistically significant increase in parenchymal β -amyloid deposition in older patients on HAART [192]. The low-level replication of HIV in the brain in patients on HAART may lead to local inflammatory production and increased APP synthesis and susceptibility to amyloid deposition. The proinflammatory cytokine environment of HIV infection in the brain could lead to increases in cytokines IL-1 α and S-100, a calcium modulating protein found in brain and systemic tissues, which are known to cause overproduction of neuronal APP [195]. Alternatively, HAART therapy itself may contribute to the overall increase in amyloid deposition. It will be important to determine the functional significance of

APP plaques in HIV-infected brains and whether other neuropathological changes associated with AD are accelerated in HIV-infected patients.

Alterations in β -amyloid metabolism have been shown to be associated with HIV infection. HIV Tat inhibits neprilysin, which degrades β -amyloid and elevates amyloid deposits in tissue culture systems [196]. The absence of neprilysin on the cell surface of neurons exposed to Tat could help explain the increase in amyloid deposits in the extracellular spaces. Tat can also interact with the low-density lipoprotein receptor and thus inhibit uptake of its ligands, including apolipoprotein E4 and β -amyloid [197]. Both β -amyloid and Tat are neurotoxic, suggesting that synergistic interactions of Tat and β -amyloid may have a role in the pathogenesis of HAD and possibly AD in HIV-infected patients.

PD represents another degenerative disorder that is likely to increase in frequency in the HIV-infected population as they age. It seems reasonable to expect the incidence of PD to increase as patients enter the age range in which idiopathic PD becomes more frequent. Impaired dopaminergic transmission is seen early and frequently in HIV/AIDS [2]. Many of the neuropathogenic pathways leading to HAD impact on the nigrostriatal system. HIV-1 Tat alters tyrosine presentin activity in mixed neuronal glial cell cultures. Whether HIV alters γ -synthase activity is unknown. The ability of L-dopa and selegiline to accelerate HIV- and SIV-related disease is likely to complicate the treatment of PD [126–128]. The effects of dopamine agonists on the course of HIV/SIV disease will be important to examine. If dopamine agonists do not accelerate HIV-related disease, they may offer the safest approach to treating PD in the HIV-infected individual. These findings suggest that HIV-infected patients may develop PD earlier and may develop more severe disease that is more difficult to treat with conventional PD agents.

24.9 CONCLUSION

Neuropharmacology offers an illuminating approach to understanding HIV-related neurological diseases. Unlike conventional viral diseases that destroy or alter cells they infect, HIV-related neurological disease is mediated through neuropharmacological pathways, including neurotransmitter systems, oxidative stress, and programmed cell death. The dopaminergic system has a central role in the pathogenesis of HIV-related neurological diseases. Neurotransmitter pathways mediated excitatory amino acid cellular cytotoxicity. The ability of several HIV regulatory proteins to form ion channels in plasma membranes and the recent demonstration of upregulation of genes encoding ion channels in the brains of HIV-infected patients with HAD illustrate the fundamental importance of neuropharmacology in the study of HIV-related diseases. The increasing importance of drugs of abuse as risk factors for HIV infection is likely to have a profound effect on the epidemiology of HIV as well as the neurological disorders encountered in HIV-infected patients. The increasing age of HIV-infected patients will likely be followed by the appearance of neurodegenerative diseases common in older age groups. The risk of accelerating HIV disease with the use of dopaminergic drugs is likely to be only one of many confounding issues that will challenge clinicians and researchers in the future. In this changing environment, the neuropharmacologist will have an increasingly important role in the study and treatment of HIV-related neurological diseases.

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LEPTIN: A METABOLIC PERSPECTIVE

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25.1	Introduction	733
25.2	Regulation of <i>ob</i> Gene Expression and Leptin Secretion	734
25.3	Leptin Receptor Expression	736
25.4	Inhibition of Leptin Signaling and Leptin Resistance	739
25.5	Leptin and Food Intake	740
25.6	Leptin and Energy Expenditure	743
25.7	Leptin and Peripheral Nutrient Utilization	744
25.8	Conclusions	748
	References	748

25.1 INTRODUCTION

The involvement of circulating factors in the regulation of energy balance was first proposed by Kennedy in the early 1950s as the lipostatic hypothesis. He argued that the careful control of body fat content in young rats was due to the hypothalamus preventing an excess in energy intake that would result in an enlargement of fat depots [1]. He subsequently suggested that the simplest way in which the hypothalamus could monitor the size of fat stores would be by sensing the circulating concentration of metabolites [2]. The presence of a circulating “feedback” signal was confirmed by a parabiosis study reported by Hervey in 1959 [3]. Parabiosis is the surgical union of two animals to produce a chronic blood exchange, and one rat in each experimental pair received ventromedial hypothalamic lesions. The lesioned rat overate and became extremely obese, whereas the intact partner had a reduced food intake and lost a significant amount of body fat. These results led to the hypothesis that fat produced a circulating “satiety signal” which informed the hypothalamus of the size of body fat depots. In the parabiotic pairs the normal rat responded to the adiposity of the lesioned partner by reducing food intake to downregulate body fat mass. Subsequent parabiosis studies, in which one member of a pair of rats was made obese by overfeeding, demonstrated that the partner of an obese rat could lose body fat even while maintaining a normal food intake [4], that the loss of fat was due to a

specific inhibition of lipid synthesis [5], and that, despite a dramatic reduction in fat, the partners of obese rats maintained a normal lean body mass [4].

Leptin was discovered by positional cloning studies [6] that were initiated as result of observations made by Coleman and Hummel [7, 8] in a series of parabiosis experiments using two different genotypes of spontaneously obese mice: diabetes (*db/db*) mice and obese (*ob/ob*) mice. Both have a phenotype that includes hyperphagia, obesity, diabetes, and infertility [9]; however, they demonstrate strikingly different responses when they are parabiosed to each other or to wild-type mice. The *ob/ob* partners of wild-type or *db/db* mice reduce their food intake and lose body fat. In contrast, food intake and obesity do not change in parabiosed *db/db* mice. These observations led to the conclusion that *db/db* mice were able to produce a circulating “satiety signal” but were unable to respond to it, whereas *ob/ob* mice did not produce the signal. In 1994 the mutation produced by the *ob* gene was identified as a failure to produce a functional form of the protein, leptin [6], and shortly thereafter the *db/db* mutation was identified as a mutation of one of the leptin receptors [10]. Based on the assumption that the mutated protein in *ob/ob* mice was the long sought-after “satiety signal,” it was hypothesized that leptin was the circulating negative-feedback signal in the regulation of energy balance [11]. Intense investigation during the past decade has shown that leptin is a pleiotropic hormone that influences many physiological processes beyond food intake and energy balance. In addition to effecting food intake through central mechanisms and modifying tissue nutrient utilization, as discussed below, leptin may function as a pro-inflammatory cytokine [12] and modulate bone remodeling [13]. Initially, leptin was thought to directly influence aspects of reproductive function; now it seems more likely that the changes in tissue metabolism and energy availability that are reflected by serum leptin concentrations play a critical role in the maintenance of reproductive function [14]. In the interest of space, in this chapter we will focus on how leptin modifies energy balance and nutrient partitioning to regulate body fat mass and maintain body composition. Because a drop in serum leptin is associated with an impairment of energy expensive processes, such as reproduction, it has been suggested that the role of leptin is to reduce energy demand during conditions of energy deficit [15]. If circulating concentrations of leptin reflect the metabolic state that is induced by an energy deficit, then low leptin levels may function to protect energy stores during periods of negative energy balance.

25.2 REGULATION OF *OB* GENE EXPRESSION AND LEPTIN SECRETION

Leptin is a 167-amino-acid peptide that is synthesized predominantly in white adipocytes but also in brown adipocytes [16], the placenta and fetal tissue [17], osteoblasts [18], mammary epithelial cells [19], glial cells of specific brain areas including the hypothalamus [20], pituitary cells [20], and gastric mucosa [21]. Investigation of the regulation of expression has focused on leptin synthesis in white adipose tissue. It is well established that circulating concentrations of leptin are positively correlated with body fat mass [22–24] and that leptin concentrations are higher in women than men with a similar degree of adiposity [25]. Expression in adipocytes is proportional to the size of the cell [26], although the slope of the relationship differs between different fat depots [26–28]. In humans, *ob* gene expression is higher in subcutaneous than omental fat [29].

Leptin release from the individual fat depots can be controlled at the level of transcription, translation, and/or secretion. The *ob* gene promoter region contains multiple functional motifs including a TATA box, multiple CAAT-enhancing binding proteins (C/EBP) and Sp1 sites, a glucocorticoid response element (GRE), several cyclic adenosine monophosphate (cAMP) response element binding sites (CREB), and LP1 [30, 31]. C/EBP are transcription factors that are critical regulators of preadipocyte differentiation. C/EBP β and C/EBP δ regulate early stages of differentiation and C/EBP α is a potent stimulator of adipocyte differentiation [32]. Therefore, it is likely that C/EBP α also is responsible for the expression of the *ob* gene during the early stages of adipocyte differentiation [32]. Sp1 transcription factor binds to GC-rich DNA elements and is involved in processes regulating cell growth. Sp1 can either activate or suppress transcription, depending upon the promoter region [33]. In the *ob* gene, Sp1 may be required for the stimulation of expression by glucose [34], discussed below. The LP1 promoter region was found to respond to an unknown factor present in adipocytes and preadipocytes but not in other cell types [31]. Physiological concentrations of glucocorticoids produce a reliable dose-response increase in *ob* gene expression that is inhibited by insulin in vitro [35]. The inhibitory effect of insulin appears to be indirect through the induction of an endogenous inhibitor of *ob* gene transcription [35]. The presence of two CREB motifs mediates inhibition of *ob* gene expression by conditions that increase intracellular cAMP concentration. Thus, activation of β_3 -adrenergic receptors [36] or melanocortin receptors [37] decreases adipocyte *ob* gene expression. It is established that estrogen increases *ob* gene expression both in vivo [38] and in vitro at the transcriptional level [39]; however, the activation appears to be dependent upon the estrogen receptor subtype present on the cell and on coactivators [40]. Administration of exogenous leptin also downregulates endogenous leptin expression [41], but the mechanism of self-regulation has not been elucidated. Although there is less information available on the regulation of *ob* gene expression in neural tissue, in vitro studies suggest that there may be some differences compared with adipocytes, because it has been reported that corticosterone decreases and dibutyryl cAMP increases *ob* gene expression in glial cells [42, 43].

Within the adipocyte, leptin is primarily stored in the endoplasmic reticulum and is transported to the plasma membrane in small vesicles [44, 45]. Although a large portion of the leptin that is synthesized is released constitutively, the presence of the vesicles allows for regulation of leptin secretion independently of transcription and translation [46, 47]. Insulin has been reported to stimulate leptin translation and secretion [47, 48] whereas isoproterenol, growth hormone, tumor necrosis factor- α (TNF α), interleukin- β 1 (IL- β 1) and cold exposure inhibit leptin secretion from adipocytes [49–52], independently of inhibiting messenger RNA (mRNA) expression. The activity of these factors may be influenced by the size of the adipocyte and/or the physiological condition of an individual because it has been reported that insulin enhances secretion of leptin from dexamethasone-treated subcutaneous adipocytes obtained from massively obese individuals [53], whereas insulin has been reported to partially reverse the dexamethasone-induced release of leptin from visceral and subcutaneous adipocytes obtained from lean and obese individuals [54].

One of the most rapid and potent regulators of *ob* gene expression is energy flux within an adipocyte. Fasting causes a substantial inhibition of expression that is

blunted in obese animals and can be prevented by maintaining blood glucose levels [55]. Expression of the *ob* gene is reversed within hours of refeeding [56], well before body fat mass changes. Similarly, there is a diurnal rhythm of adipose tissue *ob* gene expression that has been associated with the timing of meals [57]. Although the increase in leptin secretion following refeeding may be partially attributed to increased insulin secretion [58], meal feeding also increases *ob* gene expression [59], and it is clear that insulin itself does not act at a transcriptional level. There is some evidence that glucose uptake and metabolism within the adipocyte may directly regulate gene expression and leptin secretion from the cell [60] and that the fall in circulating leptin levels during fasting is inversely correlated with glucose availability [61]. It has been hypothesized that the increased flux of glucose through the hexosamine pathway in conditions of glucose abundance increases *ob* gene expression [62]. Normally, only a small fraction of glucose enters this pathway, which has an end product of uridine-diphosphoglucose-*N*-acetylglucosamine (UDP-GlcNAc) that is a substrate for glycosylation reactions [63]. Glycosylation of Sp1 may mediate the increase in *ob* gene expression that is associated with increased glucose metabolism [64]. In rats, treatments that result in an increase in tissue UDP-GlcNAc also stimulate *ob* gene expression and leptin secretion [62]. These effects can be replicated in vitro with culture of 3T3L1 cells [62] and cultured human adipocytes [65]. In vivo, UDP-GlcNAc content and *ob* gene expression are elevated in adipose tissue from obese human subjects, compared with their lean counterparts [65], but consumption of glucosamine, an intermediate for the hexosamine pathway that would also elevate UDP-GlcNAc, does not produce acute changes in serum leptin concentrations [66].

Leptin is released into the circulation in a pulsatile manner with a pulse duration of approximately 30 min. The release of leptin is exactly the inverse of adrenocorticotrophic hormone (ACTH) and cortisol [67] but cannot be attributed to regulation of release by the glucocorticoids [67]. Elevated levels of leptin in obese individuals are due to exaggerated pulse height, with no change in pulse frequency [67]. In women the pulsatility of leptin release correlates with the release of leutinizing hormone and estradiol [68], and the increased circulating concentrations of leptin in women, compared with men, also are due to increased pulse amplitude rather than frequency [69]. Based on the multifactorial regulation of *ob* gene expression and leptin secretion from the adipocyte, it is difficult to predict which factor(s) play the dominant role in expression. Because there is a good correlation between size of fat depots and leptin expression, it is likely that glucose flux and/or insulin make a significant contribution to individual baseline levels of serum leptin concentrations. This regulation likely remains intact even during the development of type II diabetes, because it has recently been shown that glucose stimulates *ob* gene expression in adipose tissue of obese, insulin-resistant mice [70]. In obese individuals, a low-grade inflammatory state [71] and increased sensitivity to glucocorticoids [54] also could contribute to elevated circulating concentrations of leptin.

25.3 LEPTIN RECEPTOR EXPRESSION

In rodents, five leptin receptors have been identified in mice and six in rats that are splice variants of a single gene [72–74]. The receptor is characteristic of a class I cytokine receptor with a single membrane-spanning domain and has homology to

IL-6, leukemia inhibitory factor, and granulocyte colony-stimulating factor receptors [72]. All leptin receptors have a common extracellular domain at the amino terminus; five receptors (ObRa–ObRd) have a transmembrane domain and an intracellular domain of varying length [72]. The intracellular domain of the receptors varies from 303 amino acids in ObRb to less than 50 amino acids in the short-form (ObRa, ObRc, ObRd, ObRf) receptors. The receptors exist as dimers and leptin binding causes conformational changes that initiate signaling [75].

ObRb signals through activation of janus kinase (Jak) and phosphorylation of tyrosine residues on the receptor. Downstream signaling includes some proteins that are common to insulin and cytokine signaling, including insulin receptor substrates (IRS1 and IRS2), growth factor receptor binding 2 (Grb-2), extracellular signal-related kinase (ERK), and signal transducer and activator of transcription (STAT) proteins [76–78]. Deletion analysis of the intracellular domain of ObRb has shown that the box 1 motif in addition to amino acids 31–36, which immediately follow the alternate splicing site for short-form receptors, are essential for activation of Jak2 [79] and that two phosphorylated tyrosine residues (Tyr⁹⁸⁵ and Tyr¹¹³⁸) contribute to signaling by ObRb [78]. Phosphorylation of Jak2 is responsible for activation of IRS proteins; both Jak2 and Tyr⁹⁸⁵ contribute to activation of ERK via an SH2-containing tyrosine phosphatase (SHP-2) [80] whereas Tyr¹¹³⁸ mediates STAT3 binding [81]. Studies with transgenic mice have shown that Tyr¹¹³⁸/STAT3 activation is essential for stimulation of energy expenditure by leptin [82] and for suppression of food intake via induction of melanocortins (see below), but it does not influence fertility or linear growth [83]. In contrast, loss of IRS2 function or blockade of inositol triphosphate kinase (PI3K) activity *in vivo* results in hyperphagia due to the inability of leptin to down regulate neuropeptide Y [84, 85] (see below). Activation of PI3K also has been implicated in the opening of adenosine triphosphate (ATP)-sensitive K channels in isolated pancreatic islets [86]. Thus it is anticipated that the development of additional selective knockouts will reveal more details of the specific relations between leptin receptor signaling proteins and aspects of leptin bioactivity *in vivo*. This information also will reveal which leptin responses are dependent on STAT3 activation and transcriptional events and which result from more rapid and direct responses, such as opening of ion channels.

Leptin receptors are expressed in most tissues, but the relative concentration of each receptor varies between tissue types. In the brain, leptin receptors are expressed at high concentrations in the arcuate nucleus and median eminence of the hypothalamus, the hippocampus, the choroid plexus, and the cerebellum [87, 88]. ObRa is the most abundant receptor present in the brain and is expressed either at higher levels than ObRb or at similar levels in areas where ObRb is at its most dense, such as the dorsomedial, ventromedial, and arcuate nuclei of the hypothalamus and the cerebellum and thalamus. [89]. Short-form leptin receptors are abundant in most peripheral tissues whereas ObRb is present at low concentrations except in the lung, adrenal, pancreas, and kidney [90, 91]. A majority of interest in leptin receptor signaling has focused on ObRb because this is the only receptor with an intracellular domain that includes tyrosine residues. All of the short-form leptin receptors retain the box 1 motif and 31–36 amino acids that are essential for Jak2 activation [73]; therefore, although it has been suggested that the short-form receptors simply function as transport proteins [92], it is possible that they also have additional biological function. This is supported by observations that *db/db* mice, which are

deficient in ObRb [93], show an increased rate of growth, changes in insulin sensitivity, and increased expression of ERK in response to leptin infusion [94], and by in vitro studies which show activation of IRS1 and ERK by leptin in cells transfected with ObRa [92].

Identification of the factors that regulate expression of membrane-bound leptin receptors is continuing to evolve. An estrogen response element (ERE) has been identified in the promoter region of the leptin receptor [95], accounting for a well-established regulation of hypothalamic leptin receptors by estrogen [96, 97]. Daily leptin injections decrease hypothalamic expression of ObRb mRNA in *ob/ob* mice [98], which are leptin deficient and normally have elevated levels of receptor expression [99]. The inhibition by leptin has been confirmed in neuroblastoma cells [100]; in contrast, fasting increases hypothalamic receptor mRNA expression and leptin binding [98, 101, 102]. The change in expression appears to be selective for the arcuate nucleus of the hypothalamus and may reflect a response to changes in circulating leptin concentrations [102]. Nutritional regulation of hypothalamic receptor expression is absent in diet-induced obese (DIO) rats independent of circulating leptin concentrations [103], either because the rats are leptin resistant or because fasting does not cause a great enough decrease in leptin concentrations. Although the change in expression would be consistent with the classic upregulation of a receptor in conditions of low ligand availability, it is surprising that leptin sensitivity would be increased during conditions of energy deficit. In contrast, continuous delivery of leptin in the periphery from miniosmotic pumps has been reported to increase ObRb mRNA expression in liver and hypothalamic tissue [104, 105] and ObRa in liver [104]. Whether these differences are due to the method of leptin delivery, the specificity of tissue expression, or receptor analysis remains to be determined. One study reported a transient increase in leptin receptor expression in the hypothalamus of high-fat-fed mice [106], whereas others failed to find a change in mRNA expression [105, 107] but found a decrease in receptor protein concentrations [108]. Thus there is evidence that leptin and/or energetic status of an animal can regulate expression of the leptin receptor, but the tissue specificity of the response remains to be defined.

A soluble form of the receptor (sObR) can be synthesized as a splice variant (ObRe) in rodents but also may be formed by shedding of the extracellular domain of membrane-bound receptors. Humans do not express ObRe mRNA [73] and sObR protein is derived exclusively from the shedding of both long- and short-form, membrane-bound receptors [109]. sObR is the primary leptin binding protein in the circulation [110] and may regulate leptin activity by preventing the protein from binding to membrane-bound receptors [111] but also may extend the half-life of leptin by delaying clearance. The delayed clearance would possibly explain why there are situations of dissociation between circulating leptin concentrations and cellular *ob* gene expression. Binding to sObR also could extend the biological half-life of leptin by providing a pool of slowly released protein [112]. ObRe is expressed at extremely high levels by the placenta in mice, resulting in elevated circulating concentrations of leptin during pregnancy [110, 113]. Circulating levels of sObR remain elevated in the neonate and then decline during development to adolescence, showing an inverse relation with indices of growth and sexual maturation [114, 115]. Thus, the high levels of sObR may inhibit leptin activity and reserve energy for growth. In adults, men have higher circulating concentrations of sObR than women and adiposity decreases sObR further [116, 117]. The reduced concentrations of

sObR in obesity and diurnal changes in circulating sObR, which are the reverse of those of leptin, may reflect inhibition of sObR by leptin [116]. Leptin decreases and sObR increases in response to calorie restriction, and individuals described as anorexic have high levels of sObR that decline as they refered and recover [118]. In contrast to the observations in humans, suggesting that leptin downregulates sObR, a recent report indicates that pharmacological levels of exogenous leptin promote sObR production in mice through stimulation of expression of membrane-bound leptin receptors and shedding of the ectodomain [104]. Additional work with mice demonstrates that fasting and food restriction increase circulating sObR, consistent with the human literature, and that the liver is the major site of sObR production [104]. One possible explanation for the increased release of sObR in the leptin-treated mice is that the high doses of leptin almost totally inhibit endogenous leptin expression and cause a massive depletion of fat stores. Therefore, it is possible that production of sObR is inversely related to endogenous leptin expression or that it is associated with the energy status of the cell, similar to the regulation of leptin expression. [104]. This concept is supported by the observation that large doses of leptin cause an acute downregulation of leptin receptors in rat liver [119].

25.4 INHIBITION OF LEPTIN SIGNALING AND LEPTIN RESISTANCE

Inhibition of leptin signaling is important from two perspectives. The first is in the quenching of signaling to prevent hyperactivity of ligand-bound receptors and the second is understanding the failure of leptin to function effectively in conditions of obesity. Several feedback signals that regulate signaling by the long-form leptin receptor have been identified. The first is suppressor of cytokine signaling 3 (SOCS3), which is recruited through activation of STAT3 by ObRb [120] and by prolonged activation of Tyr⁹⁸⁵ [81]. SOCS3 inhibits leptin signaling by binding to Tyr⁹⁸⁵ and preventing downstream events initiated by phosphorylation of this site [78]. Inhibition by SOCS3 may be attenuated by activation of SHP2, which competes for binding at Tyr⁹⁸⁵ [80]. SOCS3 can play only a contributory role in regulating leptin signaling because *ob/ob* mice are hypersensitive to leptin [41] despite overexpression of SOCS3 [120]. Protein tyrosine phosphatase 1B (PTP1B) also has been identified as an inhibitor of leptin signaling. PTP1B dephosphorylates tyrosine residues and contributes to the regulation of insulin signaling [121]. It is expressed in hypothalamic regions containing ObRb and dephosphorylates Jak2 [122]. Its role in regulating leptin signaling is supported by the observations that PTP1B knockout mice are hypersensitive to leptin [122] and that PTP1B inhibits leptin-induced activation of STAT protein in vitro [123]. Leptin signaling is terminated by the internalization of ligand bound to ObRa or ObRb in clathrin-coated pits and lysosomal degradation [124]. Internalization is driven by amino acids 8 and 29 of the intracellular domain of the receptor, which are present on both long- and short-form receptors [124], but the long-form receptors appear to be internalized faster than short-form receptors [125]. The internalization of receptors means that leptin reduces the number of available binding sites on the membrane [124]. On a less immediate time frame, leptin may contribute to regulation of its own signaling by influencing the expression of both leptin and leptin receptor mRNA [98].

When leptin was first identified, there was considerable excitement about the potential for development of effective weight loss treatments. Unfortunately, clinical trials demonstrate minimal efficacy of leptin [126], although preparations with long half-lives still have the potential to promote weight loss [127]. The limited effect of leptin on body weight has been attributed to leptin resistance [128], although the mechanistic basis of the resistance has not been clearly elucidated. These results may have been predictable based on the early observations that serum leptin concentrations increase in proportion to body fat mass [22]. This means that endogenous leptin is not able to either inhibit accumulation of body fat or to reduce existing fat depots. Animal studies have replicated the development of leptin resistance in obese or high-fat-fed rodents which have been reported to initially become resistant to leptin administered peripherally and then resistant to centrally administered protein [129, 130]. The development of leptin resistance in animal models is less reliable than that in humans and may be influenced by sex, age, housing, and method of leptin administration [131]. The early development of resistance to peripherally administered leptin has been attributed to a failure of leptin to cross the blood–brain barrier and activate hypothalamic receptors to inhibit food intake or increase energy expenditure [129]. This concept is supported by observations that leptin transport is saturated at normal circulating concentrations of leptin [132] and that spinal fluid leptin concentrations do not increase in proportion to circulating leptin [128]. In addition, the high concentrations of short-form leptin receptors in the choroid plexus are suggested to function as leptin transporters [72] and leptin transport across the blood–brain barrier is reduced in Koletsky rats, which are lacking a functional short-form receptor [133]. Increased circulating concentrations of triglyceride or extended periods of fasting inhibit blood–brain transport of the protein [134, 135], whereas adrenergic agonists increase transport [136]. Although the failure to transport leptin is an attractive hypothesis, diet-induced obese rats that are leptin resistant have elevated ObRa expression, which would suggest an increased capacity for leptin transport. In addition, Grill et al. [137] have established that leptin receptors located in the brain stem, which are not downregulated by leptin [138], are capable of modulating food intake and energy expenditure and access to leptin at these nuclei would be influenced to a lesser degree by the blood–brain barrier than at nuclei in the forebrain.

Whether or not leptin transport across the blood–brain barrier accounts for central resistance to peripheral leptin, it does not account for a failure of leptin to act directly on peripheral tissues. It has recently been reported that the livers of DIO rats are resistant to leptin-induced mobilization of lipid [139]. This suggests that changes in peripheral metabolism result in either a downregulation of leptin receptors or a postreceptor inhibition of signal transduction. Because some aspects of leptin signaling are mediated by proteins that are common to insulin signaling [81], it is possible that factors that induce peripheral insulin resistance also induce peripheral leptin resistance. It remains to be determined whether “leptin-resistant” animals that do not show energetic responses to leptin are also resistant to other aspects of leptin physiology [140].

25.5 LEPTIN AND FOOD INTAKE

The importance of central ObRb leptin receptors in the control of food intake is convincingly demonstrated by the extreme obesity that develops in *db/db* mice that

are deficient in ObRb [10] and in mice in which neural ObRb is selectively deleted [141]. The dependence of leptin-induced anorexia on activation of central leptin receptors is also supported by studies that show a more potent effect of central than peripheral leptin on food intake and adiposity [142, 143], and this difference is exaggerated in obese animals [144, 145]. Because the majority of leptin is produced by white adipose tissue, it is assumed that peripheral leptin crosses the blood–brain barrier to activate central ObRb [146]. This would be consistent with the initial supposition that leptin is the “satiety signal” demonstrated by parabiosis studies [6]. Little has been done, however, to confirm or examine the importance of leptin that is synthesized in neural tissue [147].

As described above, leptin receptors are expressed at relatively high concentrations in several hypothalamic nuclei that have been demonstrated to play a critical role in the control of food intake and/or energy expenditure. The cells in these nuclei also express neuropeptides that are known to either increase or decrease food intake [148]. Systemic administration of leptin in rats induces Fos-like immunoreactivity in many areas of the brain, including the caudal dorsomedial nucleus of the hypothalamus (DMH), the dorsal ventromedial nucleus of the hypothalamus (VMH), the superior lateral parabrachial (sellPB), subnucleus the parvicellular subdivisions of the paraventricular nucleus (PVN) of the hypothalamus [149], and the arcuate nucleus (ARC) of the hypothalamus [150]. These observations indicate that peripheral, or central, leptin can activate a number of nuclei that are implicated in the control of food intake and activation of the autonomic system. In addition, because there are projections between different hypothalamic nuclei, it also is possible that the response to leptin is modulated by indirect activation of interconnected nuclei [151].

The ARC of the hypothalamus has been a primary focus of attention because it contains leptin receptors on cell bodies that project to other hypothalamic nuclei, such as the PVN, that integrate efferent signals from various sites to modulate food intake (see Fig. 25.1). In the ARC, ObRb is located on cell bodies that colocalize pro-opiomelanocortin (POMC), the precursor of the melanocortin, α -melanocyte-stimulating hormone (α -MSH) [152], and cocaine and amphetamine-regulated transcript (CART) [153]. They also are present on neurons that colocalize neuropeptide Y (NPY) [154] and agouti-related protein (AgRP) [155], an endogenous antagonist of melanocortin receptors [156]. Leptin inhibits expression of the orexigenic peptides NPY and AgRP but stimulates expression of the anorexigenic peptides POMC and CART. As noted above, different aspects of the postreceptor signaling pathway for leptin are responsible for the activation of α -MSH and inhibition of NPY [82].

CART is a potent inhibitor of food intake that is expressed at high levels in the ARC of fed animals and is reduced to low levels during fasting [153]. CART mRNA expression also is reduced in leptin-deficient *ob/ob* mice but is increased in response to leptin administration [153]. α -MSH has a high affinity for melanocortin receptor 4 (MC4R) and to a lesser extent MC3R. These receptors are widely distributed both in the periphery and in brain nuclei that include the PVN, the lateral hypothalamus, and the DMH [148]. Agonism of MC3R and MC4R results in negative energy balance due to both inhibition of food intake and increased energy expenditure [157]; the increase in expenditure may be the result of stimulating sympathetic outflow [158]. In contrast, AgRP, which antagonizes melanocortin receptors, initiates a prolonged stimulation of food intake [159] and a decrease in energy expenditure [160] when administered centrally. It is expressed at high levels in leptin-deficient *ob/ob*

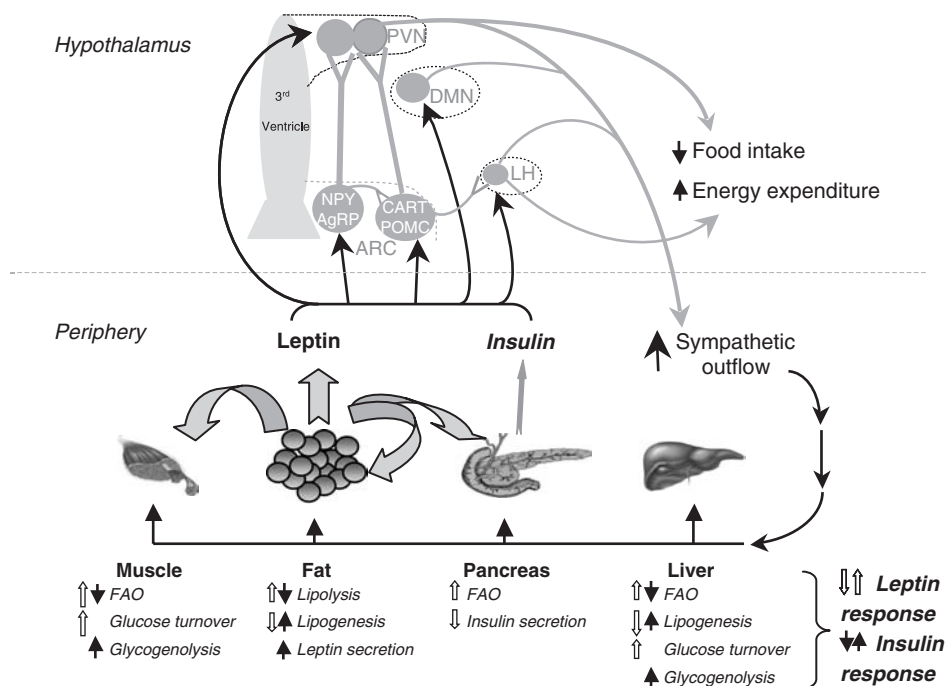


Figure 25.1 Metabolic response to leptin in peripheral tissues. Some responses may be mediated by a direct effect of leptin on the tissue whereas others result from central activation of the sympathetic nervous system. Insulin works in opposition to leptin in the periphery, but the two hormones initiate similar responses through activation of their respective receptors in hypothalamic nuclei. FAO = fatty acid oxidation.

mice and during fasting but is downregulated by leptin [155]. Although α -MSH and AgRP play a significant role in the energetic response to leptin administration, the metabolic and feeding responses to centrally administered leptin are not exclusively dependent upon melanocortin pathways because many of leptin's physiological effects are intact in *ob/ob* mice that are treated with both leptin and the melanocortin receptor antagonist SHU9119 [161]. In addition the selective reduction of body fat in mice infused peripherally with leptin is intact in animals that overexpress an endogenous antagonist of the melanocortin receptors [162].

NPY is one of the most potent orexigenic neuropeptides [163] and its expression increases in conditions of energy deficit [164]. Acute central injections of NPY stimulate food intake for several hours [163] and significantly reduce brown fat uncoupling protein (UCP) levels [165]; however, careful measures of energy expenditure indicate that there is an overall increase in energy expenditure following NPY injection due to increased activity [166] which may be representative of foraging activity [167]. The increase in expenditure is small in comparison to the increase in food intake; therefore, repeated or continuous infusion of NPY results in hyperphagia and obesity [168]. Peripheral leptin administration inhibits NPY mRNA expression in the ARC [169] but not in extrahypothalamic NPY nuclei, such as the amygdala [170]. Similarly, leptin reduces hyperphagia induced by acute central injections of NPY [171] and blunts the increase of food intake and NPY mRNA

expression associated with fasting in normal weight but not genetically obese Zucker rats, which have a mutation of the leptin receptor [142, 172]. The NPY neurons appear to become resistant to leptin with chronic exposure because an initial downregulation of NPY mRNA expression and a suppression of food intake are both reversed in rats receiving chronic third-ventricle infusions of leptin despite an apparent sustained activation of the leptin receptor signaling pathway, as indicated by Jak/STAT activation [173].

Recently there has been increasing interest in the integration of activity of different peptides in the ARC in response to peripheral metabolic status [174]. The ARC cells that express leptin receptors also respond to the peripheral hormones insulin, ghrelin, and cholecystikinin (CCK). Particular attention has been paid to the interaction between insulin and leptin signaling in the ARC because of the commonality of postreceptor signaling proteins and ion channels [175]. The interactions between insulin and leptin in the regulation of glucose utilization in peripheral tissue is discussed elsewhere in this chapter, but there also is some evidence that the two hormones act interdependently on cells in the hypothalamus [176]. This is not surprising considering both hormones are present in the circulation at concentrations that reflect both adiposity and glucose metabolism. The most convincing evidence that insulin and leptin can interact to regulate metabolism and food intake come from studies in which leptin administration restores normophagia, normoglycemia, insulin sensitivity, and reproductive function in streptozotocin, insulin-deficient diabetic rats [177–180]. The normalization of these animals is associated with a downregulation of hypothalamic AMP-activated protein kinase (AMPK) [181], a kinase that is usually increased in conditions of energy deficit and is suppressed by leptin, insulin, glucose, and positive energy balance [182]. AMPK increases fatty acid oxidation and appears to play an important role in leptin action in peripheral tissue (see below), but also it has been suggested that hypothalamic AMPK acts as a “fuel gauge” by modulating food intake in response to nutrients and metabolites that represent changes in energy balance [182].

Although much of the work elucidating leptin’s anorexigenic activity has focused on the melanocortins, NPY and insulin, leptin receptors are expressed on cells that also express other neuropeptides that are known to influence food intake and/or energy balance. For example, leptin receptors are found on cells in the lateral hypothalamus that express the orexigenic peptides melanin-concentrating hormone (MCH) [183] and orexins [184] and on glucagon-like peptide 1 (GLP1) expressing cells in the brain stem [185]. Interestingly, several studies have demonstrated a synergistic effect of leptin or insulin with CCK on food intake [186–189]. These observations may represent some of the first evidence of the integration of short-term signals of satiety with factors that are thought to carry information on long-term adiposity and energy balance. This integration of information would provide a system in which responses to changes in body energy are transformed into changes in feeding behavior.

25.6 LEPTIN AND ENERGY EXPENDITURE

In normal animals, central administration of leptin decreases body weight and adiposity both by inhibiting food intake [142] and by increasing thermogenesis [190].

The observation that leptin-treated animals lose more weight than pair-fed controls [191] provides indirect evidence of an increase in energy expenditure. Direct evidence comes from studies in which a sustained increase in heat production has been demonstrated in rats receiving repeated daily central injections of leptin [190] or a continuous peripheral infusion of a high concentration of leptin [192]. The increase in expenditure is associated with increased mRNA expression of UCP1 in brown adipose tissue (BAT) [192], which is mediated by a leptin-induced activation of the sympathetic nervous system (SNS) [193]. The activation of the SNS by leptin appears to be organ specific, because increased activity has been demonstrated in BAT, kidneys, adrenal glands, hind limb, [193] and white adipose tissue [194] of anesthetized rats, but there is no effect on cardiovascular function [193]. Most of the studies examining the effect of leptin on sympathetic outflow have been conducted in animals receiving relatively large doses of leptin; therefore it remains to be determined how much impact the normal daily fluctuations in circulating leptin concentrations have on thermogenesis. It is possible, however, that the fall in circulating concentrations of leptin during periods of energy deficit facilitates a decline in basal metabolic rate, both by removing a stimulus of thermogenesis and by downregulation of the hypothalamic–pituitary–thyroid axis, which also stimulates thermogenesis, possibly through activation of the SNS and increased expression of UCP [195]. Leptin may regulate release of thyroid-regulating hormone directly at the PVN [196] or, indirectly, through other neuropeptides such as the melanocortins [197].

In addition to the stimulation of the SNS by leptin there is substantial evidence that activation of the SNS regulates leptin *ob* gene expression. Central and peripheral leptin administration increases, sympathetic outflow [192, 193] and circulating concentrations of norepinephrine [198]. At the same time, *ob* gene expression is downregulated following sympathoadrenal stimulation by acute cold exposure, norepinephrine administration, or administration of the β -adrenergic receptor agonist isoproterenol [52]. Activation of the β -adrenergic receptors in white fat increases intracellular cAMP, which in turn would inhibit leptin production at the transcriptional level [199]. There also is in vitro evidence that activation of the adrenergic receptors inhibits both basal and insulin-stimulated leptin secretion from the adipocyte [200, 201].

25.7 LEPTIN AND PERIPHERAL NUTRIENT UTILIZATION

Although leptin administration decreases food intake and increases energy expenditure, the changes in tissue nutrient metabolism that are induced by leptin do not reflect those that are typical of food restriction. The most striking aspect of leptin action is the specificity of response in adipose tissue. Leptin-treated mice [41] and rats [202] lose a large portion of their body fat but retain lean tissue. A similar small but selective loss of body fat has been reported for calorically restricted human subjects treated with leptin [126]. This change in body composition directly replicates the changes observed in parabiotic partners of obese rats [3, 4] and supports the notion that leptin is the circulating “satiety factor” identified by these classic studies. It is important to note that leptin can reduce body fat without a significant change in food intake [41], indicating a change in nutrient partitioning, as opposed to a simple energy deficit. Although the primary change in body composition of leptin-treated

animals is a reduction in fat mass, there are changes in glucose and fatty acid utilization in liver and muscle in addition to adipose tissue. These changes are summarized schematically in Figure 25.1. Some of the changes in metabolism appear to be in response to direct activation of leptin receptors in the tissue, but other metabolic effects may be secondary to changes in neural input or the hormonal environment of the leptin-treated animal.

There appears to be a complex interaction in the periphery between leptin and insulin. As described above, insulin stimulates leptin secretion from adipocytes [47] and there is conflicting evidence on whether leptin promotes or inhibits insulin secretion from pancreatic islets. Both ObRb and ObRa are expressed on pancreatic β cells, with ObRa present as the dominant isoform [203, 204]. In vitro studies show a rapid inhibition of insulin secretion from perfused rat pancreas associated with a decrease in intracellular calcium concentration [205] and an inhibition of insulin mRNA expression [206]. The leptin response is more dramatic in hyperglycemic than normoglycemic conditions [206–208], when elevated glucose concentrations stimulate islet expression of both leptin and leptin receptor mRNA and increase the leptin sensitivity of the cells [209]. The increased mRNA expression has been attributed to increased glucose flux through the hexosamine pathway in the islets [209], a pathway which has been associated with the development of insulin resistance in muscle, liver, and adipose tissue [63, 210, 211] and which may represent a mechanism by which energy utilization is controlled in situations of energy excess. The increased leptin sensitivity of the islets in this condition has the potential to be protective at a time when intracellular lipid is likely to be elevated, because leptin promotes fatty acid oxidation and stimulates expression of UCP2 within the islets [212], both of which help to maintain functional integrity of the cells.

Leptin applied to isolated islets activates ATP-sensitive potassium channels (KATP) [213], depolarizes the cell, and decreases intracellular calcium ion concentrations. The activation of the KATP channels is dependent upon activation of PI3K, phosphodiesterase B, and cAMP [86, 214] and may involve transcriptional regulation of PI3K [215]. Leptin also activates ERK [216], which could account for the promotion of proliferation of fetal islet cells by the hormone. Administration of exogenous leptin to experimental animals produces a response similar to that observed in in vitro studies. Hyperglycemia is reversed in leptin-deficient *ob/ob* mice with doses of leptin that do not produce a significant weight loss [217] and leptin injection improves glucose clearance in rats during a hyperinsulinemic clamp [218]. In contrast, insulin release in response to a glucose challenge is exaggerated in wild-type mice 2 h after a leptin injection but is blunted in mice infused with low doses of leptin for five days [219]. The in vivo studies do not differentiate between direct effects of leptin on peripheral tissues and indirect effects mediated by central leptin receptors; therefore, it is possible that the improved insulin sensitivity of leptin-treated animals is due to central activation of sympathetic nerves, resulting in increased glucose uptake in cardiac and skeletal muscle and in brown fat but not white fat [220]. The increased glucose uptake also may be through insulin-independent pathways because central or peripheral leptin administration reverses hyperglycemia in insulin-deplete streptozotocin diabetic rats [179, 221].

In contrast to the experimental studies, there is a positive correlation between baseline circulating concentrations of insulin and of leptin in lean and obese or diabetic children and adults [222–225], implying an inverse correlation between leptin

and insulin sensitivity. This also is supported by demonstration of an inverse relation between the diurnal changes in serum leptin concentrations and insulin sensitivity in subjects subjected to a hyperglycemic, hyperinsulinemic clamp for 72 h. [226]. Although these observations are in direct opposition to the effect that leptin has on islet insulin release in vitro [213], they may be explained by the difference in duration of exposure to leptin because, in vitro, leptin initially inhibits insulin secretion from islets, but after prolonged exposure leptin increases insulin secretion [227].

The effect of leptin on glucose utilization tends to be tissue specific and seems to be controlled predominantly by leptin-induced activation of sympathetic outflow to the tissue. Central or peripheral infusion of leptin has been shown to increase hepatic glucose turnover, reduce liver glycogen content, and double whole-body glycolysis [228]. Central infusion of lower doses of leptin do not change glycolysis but inhibit hepatic glycogen synthesis and stimulate gluconeogenesis secondary to activation of the rate-limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK), indicating that many of the hepatic metabolic responses are mediated by sympathetic outflow to the tissue [229], possibly via activation of the melanocortin system [230]. When rats are infused with leptin for periods of days, rather than hours, the changes in metabolism result in reduced adiposity and enhanced insulin action, compared with pair-fed rats. Whole-body and liver glycogen synthesis is increased, hepatic glycolysis is increased, but glucose production is substantially reduced due to almost total inhibition of glycogenolysis despite increased PEPCK expression and gluconeogenesis [231]. Thus, chronic treatment with leptin simultaneously promotes glucose oxidation and maintenance of glycogen stores while inhibiting lipid accumulation in adipose tissue, and this metabolic condition does not appear to be dependent on sympathetic regulation of metabolism [232].

Similar to glucose metabolism in liver, there is little evidence for a direct effect of leptin on glucose metabolism in skeletal muscle or adipose tissue [233, 234]. In vitro, leptin stimulates glycogenesis in muscle from *ob/ob* mice [235], which are hypersensitive to leptin [41], but has no effect in tissue from wild-type mice or rats [233, 235]. In contrast, muscle glucose uptake and glycogen content are increased in animals that receive central or peripheral infusions of leptin [228, 236], due to activation of central leptin receptors [228]. In vivo administration of physiological doses of leptin causes a rapid (3-min) increase in adipose STAT3 phosphorylation and activation of mitogen-activated protein kinase (MAPK), with only a small initial effect on PI3 K activity [237], indicating a direct effect of the hormone on adipose ObRb. In isolated adipocytes, leptin has been reported to inhibit the incorporation of glucose into lipids [238] and to stimulate glucose oxidation [239]. Other studies have not replicated these results [219, 240, 241], but there is a reliable inhibition of glucose utilization [242] and lipogenesis in adipose tissue taken from animals treated with leptin in vivo [219]. One of the reasons for the discrepancies between studies is that leptin has been shown to interfere with insulin binding to its receptor [243]; therefore, it is difficult to separate which responses are a direct effect of leptin and which are secondary to inhibition of insulin action.

In addition to modifying insulin sensitivity and glucose utilization by peripheral tissues, leptin shifts lipid metabolism away from lipogenesis and toward fatty acid oxidation (see Fig. 25.1). Elevated circulating concentrations of leptin in rats cause a rapid and dramatic reduction in the triglyceride content of liver, adipose tissue, skeletal muscle, and pancreatic islets [244, 245] due to activation of local leptin

receptors. For example, physiological doses of leptin produce a 42% reduction in the triglyceride content of isolated, perfused livers within 90 min [139]. The reduction in tissue lipid is associated with an inhibition of lipogenesis and activity of hepatic stearyl-CoA desaturase-1, an enzyme involved in fatty acid synthesis [246], and a substantial elevation of enzymes associated with fatty acid oxidation [212]. Activation of muscle ObRb does not lead to activation of STAT3, MAPK, or PI3K [237] in skeletal muscle, but recently, a key role for AMPK, an enzyme that promotes fatty acid oxidation by inhibiting acetyl coenzyme-A carboxylase (ACC) [247], has been identified. ACC normally increases the activity of malonyl-CoA, which in turn inhibits fatty acid oxidation. Leptin treatment leads to an increase in the intracellular AMP-ATP ratio through an as-yet-unidentified signaling pathway, and the resulting phosphorylation of AMPK stimulates fatty acid oxidation. In muscle, leptin initially (minutes) activates AMPK via muscle leptin receptors, but over the longer term (hours) AMPK is stimulated by leptin-initiated sympathetic activation of α -adrenergic receptors on the muscle tissue [245]. The reduction in intracellular lipid content of nonadipose cells can potentially protect against the development of diabetes and metabolic syndrome because the accumulation of long-chain acyl CoA disrupts insulin release and insulin sensitivity [248, 249].

The selective reduction of body fat in leptin-treated animals can potentially be achieved either by inhibiting lipogenesis and lipid accumulation or by promoting lipolysis and lipid catabolism. There is conflicting evidence on whether leptin promotes lipolysis in adipose tissue. Extremely high concentrations of leptin have been shown to increase glycerol release from isolated adipocytes [250, 251] and to increase circulating concentrations of glycerol or fatty acids in vivo [252, 253]. Other studies have failed to find a significant effect of leptin on adipocyte glycerol release [254, 255]. These latter observations would be consistent with the metabolic changes in lean parabiotic partners of obese rats, in which the loss of body fat was associated with a significant inhibition of adipose lipogenesis but no change in glycerol release until body fat was greatly diminished and lipolysis also declined [256]. There is more convincing evidence that the reduction of body fat in leptin-treated animals is caused by an inhibition of lipid synthesis, similar to the changes in parabiotic rats [256]. Single injections and peripheral infusions of low doses of leptin for 5 days cause a significant inhibition of adipose tissue lipogenesis in mice [219, 257]. If these changes in metabolism are caused by a direct activation of leptin receptors on adipocytes, then the changes in lipid metabolism may be secondary to the activation of AMPK, which would inhibit ACC and fatty acid synthesis [247] but, in addition, adipose tissue from hyperleptinemic rats show a significant downregulation of sterol regulatory element binding protein (SREBP1), a transcription factor that regulates expression of a family of enzymes including fatty acid synthase [258]. In vitro, leptin inhibits insulin-stimulated but not basal lipogenesis [240, 254, 259], which may be secondary to the inhibition of insulin binding to insulin receptors [243], and suggests that the inhibition of basal lipogenesis in vivo is caused by mechanisms other than direct activation of adipocyte leptin receptors. Although the response is indirect, leptin appears to inhibit adipose tissue lipid accumulation independent of central activation of the sympathetic nervous system. It has been reported that there is no significant effect of leptin on norepinephrine turnover, an index of sympathetic activation, in white adipose tissue [260] and this has been supported by observations that peripheral infusions of leptin reduce the size of fat pads in which sympathetic

nerves have been selectively denervated by injection of 6-hydroxydopamine [261]. In addition, there is a significant reduction in the size of fat transplants, which have no neural supply, in hyperleptinemic rats [262]. Therefore, although it is clear that leptin has a selective effect on body composition to reduce body fat but protect lean tissue, the exact mechanistic basis of this shift in nutrient partitioning and metabolism has yet to be clearly defined.

25.8 CONCLUSIONS

Early expectations that leptin would provide an effective means of treating and preventing obesity have not been met, but it is clear that leptin plays an important role in regulating many different physiological functions based on energy availability. Administration of exogenous leptin to leptin-responsive experimental animals causes a selective reduction in body fat and a beneficial reduction in intracellular lipid in insulin-responsive organs. These changes are achieved through direct activation of leptin receptors on the different cell types, modification of insulin action, and activation of sympathetic output to muscle and liver. Further studies are needed to elucidate the mechanisms that induce "leptin resistance" in conditions of obesity. Identification and reversal of these processes would then potentially provide a much needed treatment for obesity, by increasing leptin sensitivity in individuals with high circulating concentrations of endogenous leptin.

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26

GHRELIN: STRUCTURAL AND FUNCTIONAL PROPERTIES

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26.1	Introduction	766
26.2	Peptide hormone ghrelin and other ghrelin receptor ligands	766
26.2.1	Ghrelin: Acylated Peptide Hormone	766
26.2.2	Expression Pattern of Ghrelin	768
26.2.3	Growth Hormone Secretagogues and Other Ghrelin Receptor Ligands	769
26.3	Ghrelin receptor	770
26.3.1	Constitutive Activity of Ghrelin Receptor	770
26.3.2	Subfamily of Receptors Homologous to Ghrelin Receptor	771
26.4	Central effects of ghrelin	772
26.4.1	Appetite and Metabolic Regulation	772
26.4.2	GH Secretion	773
26.4.3	Central Effect on Sympathetic Nervous System	773
26.4.4	Other Central Effects	773
26.5	Peripheral function of ghrelin	774
26.5.1	Cardiovascular Action	774
26.5.2	Modulation of Adipocytes and Body Composition	774
26.5.3	Thyroid Function	774
26.5.4	Modulation of Immune System	774
26.5.5	Pancreas Function and Influence on Insulin Resistance	775
26.5.6	GI Tract Function	775
26.5.7	Bone	775
26.5.8	Antiproliferative/Proliferative Effect	776
26.6	Clinical perspective of ghrelin agonists and antagonists	776
	References	776

26.1 INTRODUCTION

Ghrelin is a recently discovered peptide hormone secreted from the gastrointestinal (GI) tract which has an important impact on the regulation of appetite and metabolism. However, the story of ghrelin started more than 20 years before the hormone was finally discovered in 1999 by Kangawa and co-workers. At this time ghrelin was expected to act mainly as a growth hormone (GH)–releasing substance, and so it is named after this function.

In the mid 1970s it was found that enkephalin-like peptides, developed in the search for analgesics, also had the ability to induce GH release from pituitary cells [1, 2]. Interestingly, this GH secretion was independent of the GH-releasing hormone receptor and the release of other pituitary hormones was not affected [2]. Encouraged by this observation, the pharmaceutical industry established large drug discovery programs in an effort to develop cost-effective GH-releasing drugs that could compete with very expensive GH treatment [3]. High potent peptide and nonpeptide compounds—collectively named GH secretagogues (GHSs)—were developed, leading to the final discovery of the receptor for these synthetic compounds in 1996 [4]. Some of the GHS compounds have been tested for the presumed GH-secreting properties in large clinical trial. However, the results were not sufficiently convincing to support further clinical development with GH secretion as the primary focus [5, 6].

It was a surprise for most researchers in the GHS field that the endogenous hormone of the GHS receptor was eventually found in the GI tract and not in the brain. The subsequent discovery of ghrelin as an important modulator of food intake and metabolism has renewed interest in the ghrelin receptor as an anticachexia target and more importantly as an antiobesity target.

26.2 PEPTIDE HORMONE GHRELIN AND OTHER GHRELIN RECEPTOR LIGANDS

26.2.1 Ghrelin: Acylated Peptide Hormone

Ghrelin is a 28-amino-acid-long peptide hormone with a unique fatty acid modification on the serine in position 3 (Fig. 26.1) [7]. The fatty acid attached by esterification is most often an octanoyl (8-carbon chain), but a minor population of endogenous ghrelin molecules are modified by a chain of 10-carbon atoms either with or without double bonds [8]. As shown in Figure 26.1 the size of the fatty acid chain appears relatively small and insignificant compared to the rest of the large peptide; however, the fatty acid is of crucial importance for binding to and functional activation of the ghrelin receptor (Fig. 26.1). Synthetic peptides where the fatty acid has been systematically changed have shown that a decrease in length of the carbon chain rapidly decreases the functional capacity of the molecule—as measured with calcium mobilization—whereas an increase in the number of carbon atoms and introduction of double bonds only affect the potency of the peptide to a limited extent [9, 10]. If the acyl group is moved to the neighboring serine in position 2, the potency is decreased more than 200-fold [10]. Interestingly, replacement of the octanoylated serine with an unmodified tryptophan or the artificial amino acid naphthylalanine (Nal) will only

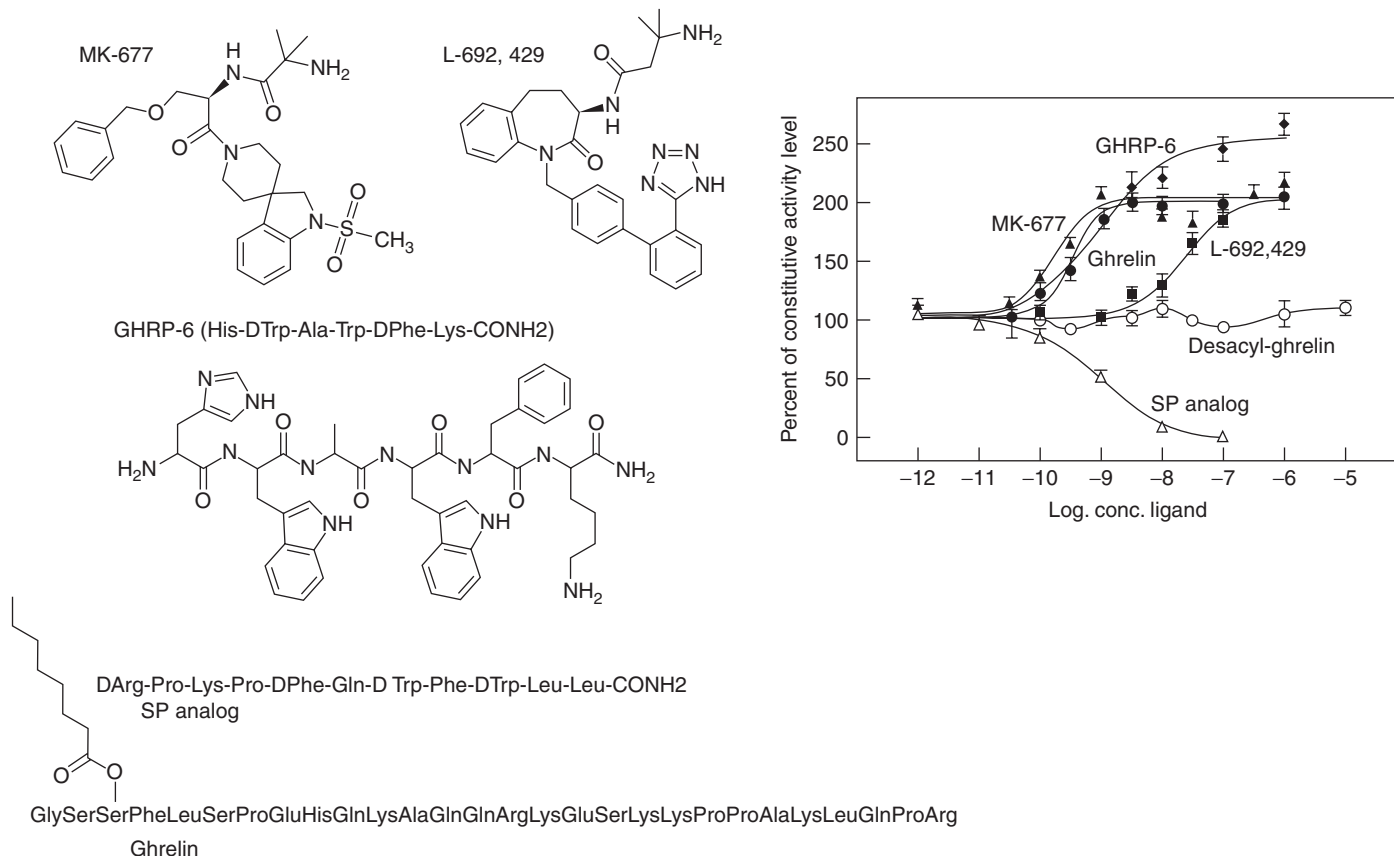


Figure 26.1 Structures and activity of ghrelin receptor ligands. L-692,429 is a benzoelactam nonpeptide compound initially identified as a GHS, based on chemical components from cholecystokinin (CCK) and angiotensin receptor ligands [3]. MK-677 is a subsequent spiroindanylpiperidine nonpeptide GHS which has been used in several clinical trials [6]. GHRP-6 was one of the original peptide-based GHSs of Bowers [2]. Ghrelin, which requires octanylation of Ser³ for most biological effects, is the endogenous peptide ligand for the ghrelin receptor [7]. [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P (SP analog) is a highly potent inverse agonist at the ghrelin receptor and a low-potency antagonist on the several different peptide receptors. The activity is measured as inositol phosphate accumulation in transiently transfected COS 7 cells and expressed as a percentage of maximal constitutive activity.

decrease the potency of the molecule a fewfold, whereas introduction of less hydrophobic amino acids like leucine and valine decreases the activity to the same level as observed for des-acyl ghrelin [9]. Acylation of a peptide has previously been described for intracellular proteins, but ghrelin is the first peptide hormone to be described with this kind of modification. The acylation modification brings up a number of important—but yet unanswered—questions. First, it is very hard to understand how the binding energy provided by such a small attachment to the peptide is sufficient to explain the 1000-fold decrease in affinity when ghrelin is deacylated. Motilin is the peptide hormone most closely related to ghrelin based on structural features and similarly the motilin receptor is closely related to the ghrelin receptor. However, motilin binds with high affinity to its receptor without modification [11]. Second, the enzyme responsible for the transfer of an acyl group to the serine hydroxyl group is not known. This enzyme may be a very important target in the development of drugs to regulate the activity of ghrelin [12]. The additional complexity brought about by ghrelin acylation and des-acylation processes may be physiologically important for providing fast regulation of hormone activity. In the future, other hormones and neurotransmitters with posttranslational modifications may be discovered and the physiological relevance of such modifications will be revealed.

Truncation of ghrelin from the carboxy-terminal end has shown that the 18 most amino terminal residues are required for full binding affinity and potency in functional receptor studies. Full potency and efficacy have been described for the five most amino terminal residues. However, this penta-peptide binds with poor affinity to the receptor [10, 13]. Substitution and deletion of residues in the middle of the 28-amino-acid-long peptide does not affect its function [9]; deletion of a central glutamine (Gln¹⁴) is a naturally occurring splice variant which has full activity [14]. Similarly, rodent ghrelin, which has two single amino acid substitutions in positions 11 (arginine to lysine) and 12 (valine to alanine), binds with almost the same affinity as human ghrelin to the human ghrelin receptor [9].

26.2.2 Expression Pattern of Ghrelin

Ghrelin was originally isolated from the stomach, which at that time was surprising since the hormone was believed to modulate GH secretion [7]. However, the endocrine cells in the stomach are responsible for secretion of a large number of neuroendocrine transmitters that modulate food intake, metabolism, and digestion [15]. Ghrelin is the first characterized hormone to be secreted from A- or X/A-like endocrine cells, located mainly in the acid-producing part of the stomach, called the oxyntic gland, with an additional scattered population in the small and large intestine [16]. The ghrelin-producing cells in the stomach are not in contact with the luminal side but are positioned close to the capillaries, indicating a function independent of the gastric content. In contrast, some of the ghrelin-producing cells in the lower part of the GI tract are in contact with the lumen. The significance of this is unknown [17].

Surgical removal of the acid-producing proximal part of the stomach or the entire stomach induces a 75% decrease in plasma ghrelin levels which slowly recovers to approximately 50% of the initial level seven days postsurgery. This does not improve further as 50% of normal ghrelin level is still observed two years after

surgical removal of the stomach [18, 19]. Thus, under normal conditions most of the circulating ghrelin appears to be produced in the stomach and the intestine. Pancreatic islets also express high levels of ghrelin, localized in α cells [20] and a novel ϵ -cell type [21]. Embryonic pancreatic expression is particularly high, indicating a functional importance for the development of the endocrine pancreas [21].

In addition to the GI tract, ghrelin expression has been observed at messenger RNA (mRNA) level in various different tissues and the ghrelin peptide has been detected in the pituitary gland, immune cells, kidney, lung, placenta, ovary, and testis and in several tumor tissues [20, 22].

26.2.3 Growth Hormone Secretagogues and Other Ghrelin Receptor Ligands

After the first metenkephalin derivatives were synthesized by Bowers and co-workers in the 1970s [1], GHRP-6 was the first hexapeptide to induce high potent GH release in vivo in both humans and animals. Despite low bioavailability and short half-life, oral administration of GHRP-6 showed strong GH-releasing effects in humans [3]. Subsequent research aiming at improved pharmacokinetic properties led to the discovery of a large number of both peptide and nonpeptide compounds, many of which have been tested in clinical trials [3, 5].

A number of the peptide GHS compounds have structural similarities. The key features are in sequence: a spacer of variable length from the amino terminal, a very hydrophobic amino acid in the D stereoisomer (e.g., D-Trp, D-Nal, or D-Me-Trp) followed by another hydrophobic residue and finally a lysine as neighbor to the amidated carboxy-terminal residue. A simplified pharmacophore has been proposed to comprise only a diaromatic core and a basic amine [3].

The first series of nonpeptide GHS, of which L-692,429 is the most well characterized, turned out to have low oral bioavailability [3]. However, members of a subsequent class of compounds, spiroindanyl-piperidines, were orally active, and from this series MK-677 (Fig. 26.1) was later investigated in several clinical trials [3, 5].

In parallel with the drug discovery programs for synthetic ligands the pharmaceutical industry attempted to find the endogenous ligand for the receptor responsible for the GHS-induced GH release. Most efforts were focused on the brain. From brain extracts two different groups found almost simultaneously that adenosine was an agonist on the GHS receptor inducing calcium mobilization in micromolar concentrations [23, 24]. However, adenosine does not induce GH secretion [24] or IP accumulation and only a small increase in cyclic adenosine monophosphate (cAMP) accumulation has been observed [25].

Synthetic somatostatin-like peptides such as lanreotide and vapreotide have been shown to displace radiolabeled GHS compounds from pituitary membranes, a finding that has encouraged similar experiments with the endogenous cortistatin [26]. It was observed that both ghrelin and cortistatin-14 displaced the radiolabeled GHS compound with an affinity of 500 nM [26]. This affinity for ghrelin is more than 100-fold lower than what is measured in heterologous expression systems, and importantly the radioligand itself was binding with an affinity at approximately 100 nM [27]. Though cortistatin has been suggested to counteract the function of ghrelin [28], convincing binding, a direct antagonist or inverse agonist effect of cortistatin on the GHS receptor, remains to be shown.

26.3 GHRELIN RECEPTOR

Labeling of the nonpeptide high-affinity ligand MK-677 with ^{35}S made it possible to successfully isolate the receptor for the GHS compounds, which later turned out to be the ghrelin receptor [4] (also called GHS-R1a). This receptor belongs to a large superfamily of seven transmembrane (7TM) receptors, whose activation elicits intracellular signaling through coupling to heterotrimeric G proteins and additional G-protein-independent pathways (e.g., through arrestin recruitment) [29].

The most frequently used assay when evaluating the ghrelin receptor has been calcium mobilization as measured by changes in aequorin or in fluorescence probes. This kind of assay is very suitable for high-throughput identification of agonists. However, calcium mobilization may be achieved through various intracellular signaling pathways. Calcium mobilization for the ghrelin receptor is generally assumed to be a downstream event of phospholipase C activation where an increase in intracellular inositol triphosphate (IP3) is responsible for the subsequent release of calcium from intracellular stores. Another possible mechanism for calcium mobilization relates to diacylglycerol (DAG)-mediated activation of protein kinase C (PKC). PKC phosphorylates tyrosines on potassium channels, which depolarizes the cell and opens voltage-dependent L-type calcium channels [3].

26.3.1 Constitutive Activity of Ghrelin Receptor

Recently it was observed that the ghrelin receptor transiently expressed in Cos 7 cells signaled constitutively through IP turnover [27], a receptor property that remained unnoticed for many years because mobilization of intracellular calcium does not reveal constitutive activity [30]. The constitutive activity was decreased by nanomolar concentration of [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P, which acted as a highly potent inverse agonist on the receptor, whereas it had 100-fold lower potency as an antagonist [27].

Gene-dosing experiments with the ghrelin receptor performed in transiently transfected human embryonic kidney (HEK) 293 cells resulted in a dose-dependent but ligand-independent constitutive stimulation of the cAMP-responsive element (CRE) pathway, as monitored by a reporter assay using CRE-driven luciferase activity. The CRE-dependent transcriptional activity could be further increased by ghrelin and decreased by the inverse agonist [D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹]-substance P [31]. Although the activity of CRE is generally believed to be controlled by cAMP-dependent kinases, it can also be activated by downstream kinases of the $G\alpha_q$ pathway such as Ca^{2+} /calmodulin kinase IV or PKC [32, 33] likely to be stimulated by the ghrelin receptor.

Transcriptional regulation through the serum response element (SRE) pathway was also activated by both constitutive and agonist-dependent ghrelin receptor signaling [31]. This pathway may be stimulated by various G-protein systems including $G\alpha_{13}$, $G\alpha_i$, and $G\beta\gamma$, respectively [34–37]. $G\alpha_{12/13}$ involving Rho kinase has been suggested to mediate the coupling of the ghrelin receptor to SRE-dependent transcription, since a specific blocker of Rho kinase decreased the ghrelin-induced SRE transcription [31].

26.3.2 Subfamily of Receptors Homologous to Ghrelin Receptor

The closest structural homolog of the ghrelin receptor is the motilin receptor, which also has a ligand very similar to ghrelin [11]. These two receptors together with the neurotensin, neuromedin U, and some orphan receptors define a small subfamily of 7TM receptors (Fig. 26.2). All members of this receptor family have an intron in the coding region between TM5 and TM6. For the ghrelin receptor two different splice variants have been identified: the functional receptor GHS-R1a, which has seven TM helices (GenBank accession no. U60179), and GHS-R1b (U60181), a receptor predicted to be truncated after the fifth TM region.

The structure of the translated mRNA fragment of GHS-R1a is shown in Figure 26.2b. Two different mRNA transcripts for GHS-R1b are possible. It has been speculated, but never shown, that GHS-R1b utilizes an alternative polyadenylation site, resulting in an approximately 2-kb-long mRNA transcript coding for GHS-R1b [38] (Fig. 26.2). In contrast, it has been shown, using RNase protection assays with poly A⁺ mRNA, that the approximately 4-kb mRNA transcript of GHS-R1b exists [39, 40]. Functional responses mediated through the GHS-R1b have never been observed; therefore most research has been focused on the functionally active 7TM splice variant, GHS-R1a. When measuring the expression level and patterns of the

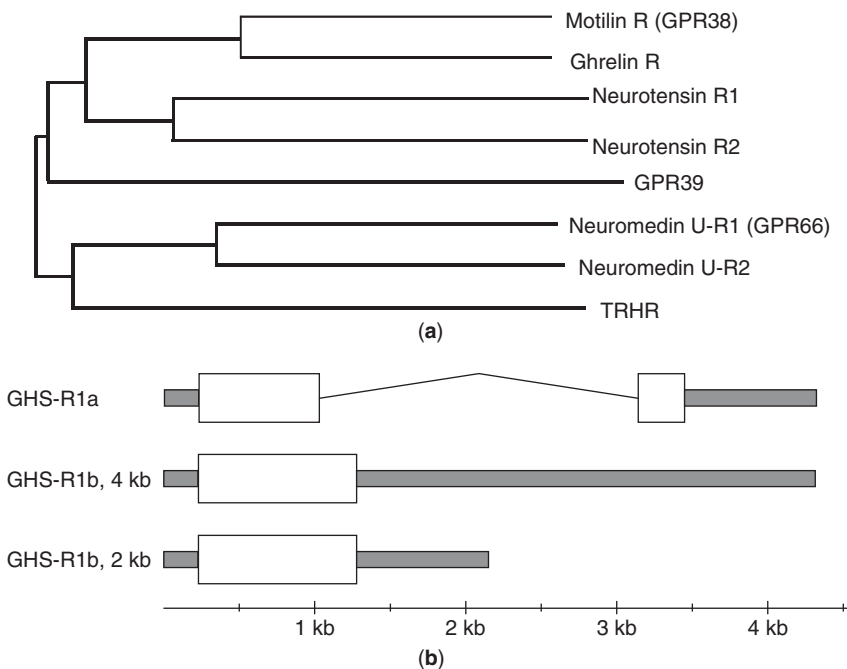


Figure 26.2 (a) Phylogenetic tree for human 7TM receptors most structurally related to ghrelin receptor. (b) Genomic structure and mRNA fragments of ghrelin receptor. The known structure of GHS-R1a is shown on the top. The two lower figures illustrate the putative structures of the mRNA fragment for GHS-R1b, which is either approximately 2 or 4 kb. The gene is located on *Homo sapiens* chromosome 3, location 3q26.31. Open boxes represent coding regions, filled boxes represent untranslated regions, the line represents an intron.

GHS-R1a receptor by use of reverse transcriptase polymer chain reaction (RT-PCR) and in situ hybridization, it is therefore important to design primers that are located on each side of the intron in order to prevent interference from the GHS-R1b mRNA transcript (Fig. 26.2). Studies using intron-spanning primers revealed expression of the functional ghrelin receptor 1a in the pituitary gland, hypothalamus, thyroid, pancreas, spleen, myocardium, adrenal, carcinoid tissues, testis, and ovary [20].

26.4 CENTRAL EFFECTS OF GHRELIN

26.4.1 Appetite and Metabolic Regulation

Increased food intake is at present the best described and apparently the most important physiological function of ghrelin. Plasma levels of ghrelin increase 1–2 h prior to a meal and then decrease shortly after the meal is initiated [41]. Ghrelin is the only known peripherally produced orexigenic (appetite-promoting) substance. The surge in plasma level, which is independent of time- and food-related cues and correlates well with hunger scores, supports the notion that ghrelin is of crucial importance for the initiation of a meal [42]. Simple distension of the stomach by water does not suppress plasma ghrelin levels. The decrease in plasma ghrelin is proportional to the energy content of a meal [43]. It has been shown that the higher the energy content of a meal, the lower is the following nadir plasma ghrelin and the greater is the delay in the subsequent request for meal [43]. Importantly, this nutrition-related suppression does not require the presence of nutrients either in the stomach or in duodenum. Thus, postprandial ghrelin regulation is mediated by more downstream intestinal signals or by postabsorptive mechanisms [44].

Locally secreted ghrelin may act either as a circulating hormone or as an initiator of activity in afferent vagal neurons [45, 46]. The vagal neurons will relay the ghrelin stimulus to centers in the central nervous system (CNS) such as the nucleus tractus solitarius which further communicate with appetite and energy homeostasis regulatory centers such as the paraventricular nucleus and arcuate nucleus in the hypothalamus. As a hormone, ghrelin acts through the blood stream on central appetite-regulating neuropeptide Y (NPY)/AgRP neurons in the arcuate nucleus, which express ghrelin GHS-R1a receptors. It has been shown in several rodent models that ghrelin administered both peripherally and centrally increases the activity and mRNA expression in the NPY/AgRP neurons [47, 48]. Furthermore, ghrelin has been demonstrated to induce a combined effect of NPY and AgRP with respect to efficacy and timing [49]. Gene knockout studies find that deletion of NPY attenuates the ghrelin-induced feeding, whereas the ghrelin-induced responses in AgRP knockout mice are normal. However, double-knockout mice AgRP(–/–)/NPY(–/–) have no detectable response to ghrelin [50]. Ghrelin may also affect the function of the pro-opiomelanocortin (POMC) neurons through inhibitory γ -aminobutyric acid (GABA)–ergic neurons [51].

Most of the ghrelin responsive neurons in the arcuate nucleus are located inside the blood–brain barrier and are consequently not accessible to blood-borne ghrelin, given that this peptide has not been demonstrated to pass the blood–brain barrier [52]. However, some POMC and NPY/AgRP neurons are found in the nearby median eminence, a circumventricular organ, situated outside the blood–brain barrier, and therefore these neurons could be targets for hormonally transmitted ghrelin from the

GI tract [52]. Additionally, a minor population of ghrelin-producing neurons which communicate with the NPY, AgRP, corticotropin-releasing hormone (CRH), orexin, and POMC neurons have been described in the hypothalamus [22, 51].

The molecular mechanism for ghrelin-induced appetite regulation in the hypothalamus may involve activation of AMP-activated kinase (AMPK). In liver and muscle cells AMPK acts as an intracellular energy sensor, switching on catabolic pathways and suppressing anabolic pathways to maintain energy levels in the cell. Ghrelin administration directly into the rat brain increases hypothalamic AMPK activation. Since a specific AMPK agonist has been shown to induce a similar increase in feeding as observed by ghrelin, it has been proposed that ghrelin acts through AMPK in the hypothalamus [53]. However, feeding and fasting do not affect hypothalamic AMPK activity [53, 54].

26.4.2 GH Secretion

Exogenous ghrelin administration acutely increases plasma GH levels, but it remains to be shown that physiologically secreted ghrelin affects the pulsatile release of GH. GHS-R1a receptors are expressed on both somatotrophic cells in the pituitary and on GH-releasing hormone (GHRH) cells in hypothalamus. Ghrelin seems to cause GH secretion at both sites, as demonstrated by an attenuation of the ghrelin-induced GH secretion by GHRH antagonist or antibody administration [55]. Plasma levels of ghrelin and GH are correlated under conditions such as fasting and sleep [56–58], but ghrelin does not affect the GH secretion during exercise or insulin-induced hypoglycemia [59, 60].

In two different case reports of patients with ghrelin-producing gastroenteropancreatic cancers, an extremely high level of ghrelin was reported without any effect on GH or insulin-like growth factor type I (IGF-I) levels [61, 62]. In contrast, administration of synthetic GHSs in pharmacological doses does increase IGF-I after chronic administration [3]. Furthermore, ghrelin receptor knockout animals have been shown to have modestly lower IGF-I levels compared to wild-type animals [63]. Consequently, the physiological interplay between GH and ghrelin remains to be clarified.

26.4.3 Central Effect on Sympathetic Nervous System

Intracerebroventricular (ICV) injection of ghrelin decreases core body temperature in rodents, indicating reduced resting energy expenditure [64]. Furthermore, central administration of ghrelin has been shown to suppress energy expenditure and thermogenesis in brown adipose tissue via an inhibitory effect on sympathetic nerve activity [65]. Increase in pancreatic polypeptide levels immediately after intravenous ghrelin administration may be indirectly responsible for the ghrelin-induced modulation of the balance in parasympathetic/sympathetic nervous system activity [66].

26.4.4 Other Central Effects

Ghrelin receptors have been detected by use of the GHS-R1a ligand MK-677 in a wide range of brain areas, including the hippocampus, thalamus, and several nuclei in the brain stem. These receptors may contribute to the few reported actions of

ghrelin on behavioral changes so far. Ghrelin is suggested as an endogenous sleep-promoting factor, as its administration increases slow-wave sleep and reduces rapid-eye-movement (REM) sleep [67]. Furthermore, it has been suggested that ghrelin induces anxiety and improves memory [65].

26.5 PERIPHERAL FUNCTION OF GHRELIN

26.5.1 Cardiovascular Action

The ghrelin receptor is highly expressed both in cardiac tissue and in blood vessels [20]. Infusion of ghrelin in healthy humans and rodents has been shown to increase cardiac output and decrease systemic vascular resistance [68, 69]. Repeated administration of ghrelin improves cardiac structure and function and attenuates cardiac cachexia in rats with heart failure [68]. These positive effects of ghrelin on cardiovascular function have encouraged studies of ghrelin administration to patients suffering from cardiac failure combined with cachexia. Recently, the results from a three-week intervention study was published showing that the patients had improved left ventricular function, exercise capacity, and muscle wasting [70].

26.5.2 Modulation of Adipocytes and Body Composition

The strongest effect of ghrelin on food intake is obtained by ICV administration. Peripheral administration of ghrelin has a less pronounced effect on food intake, despite strong effects on body weight [48]. In one study, freely fed ghrelin-treated rats showed a trend toward increased subcutaneous adipose tissue whereas pair-fed animals had a significantly increased visceral fat mass [71], indicating a feeding-independent increase in visceral fat. The administration pattern of ghrelin may also affect which outcome arises. Intermittent administration of ghrelin has been shown to strongly increase food intake, whereas continuous infusion of ghrelin has a much stronger influence on adipose tissue hyperplasia [72]. The adipogenic effect of ghrelin is probably explained by a direct inhibition of ghrelin on lipolysis and promotion of adipogenesis [73] together with an antiapoptotic and mitogenic effect on fat cells [74].

26.5.3 Thyroid Function

The ghrelin receptor is also highly expressed in the thyroid gland; however, its functional importance here remains to be elucidated [75]. It has been known that the circulating plasma levels of ghrelin are decreased in hyperthyroid patients and normalized as the thyroid parameters are normalized [76]. In contrast, patients with low thyroid function show an increase in plasma ghrelin. Furthermore, the fact that patients suffering from a ghrelin-producing tumor developed hyperthyroidism with rising thyroid-stimulating hormone (TSH) level and low T_3 and T_4 in the terminal stage [61] indicates that ghrelin may affect thyroid function.

26.5.4 Modulation of Immune System

Increasing circulating ghrelin concentrations have also been shown to elevate adrenocorticotrophic hormone (ACTH) and thus cortisol levels [77], inducing a

general suppression of the immune system. Furthermore, ghrelin may act directly on activated T cells to inhibit expression of proinflammatory anorectic cytokines such as interleukin (IL) 1β and IL-6 and tumor necrosis factor- α (TNF- α) [78]. Ghrelin leads to a dose-dependent inhibition of leptin-induced cytokine expression, while leptin upregulates ghrelin receptors on human T lymphocytes [78].

26.5.5 Pancreas Function and Influence on Insulin Resistance

Ghrelin receptors may be expressed on both endocrine α and β cells. Conflicting data have been presented with respect to the functional effect of ghrelin on pancreas [77]. On isolated pancreatic islet cells ghrelin was able to stimulate insulin secretion [79]. In contrast, other studies in vivo demonstrate that acute administration of ghrelin induces a transient decrease in spontaneous insulin secretion and selectively blunts the insulin response to arginine but not to an oral glucose load [80]. Interestingly, nonacylated ghrelin has been shown to counteract the ghrelin-induced modulation of insulin and glucose level without any effect on the neuroendocrine actions of ghrelin [81].

It is evident that ghrelin correlates with insulin levels and insulin resistance. Low plasma ghrelin levels are associated with insulin resistance both in healthy volunteers [82] and in several pathological conditions such as polycystic ovary syndrome, type II diabetes, acromegaly, and primary/secondary hypogonadism. Although the correlation in many of these conditions may indirectly arise through the close correspondence between low ghrelin levels and obesity, reduced plasma ghrelin levels are also observed in individuals with insulin resistance but normal or low body mass index (BMI), nonalcoholic fatty liver disease, and type I diabetes mellitus [83, 84].

26.5.6 GI Tract Function

Ghrelin is secreted from the GI tract and may exert local functions such as increased gastric acid secretion, gastric motility, and gastric emptying [11]. In rodent models ghrelin acts as a very potent gastropromkinetic agent that accelerates the normal emptying rate in doses compatible with those that increase food intake. However, a complicating factor is that the rodents are not a useful model for humans. Motilin, which in humans exert important prokinetic function on the GI system, does not exist in rodents, whereas ghrelin apparently is the functional equivalent in rodents [85].

26.5.7 Bone

A number of studies have shown positive effects of GHS compounds on bone. In human studies, two-month administration of MK-677 induced increase in IGF-I, bone resorption, and bone formation as assessed by type I collagen, urine hydroxyproline/creatinine, and calcium/creatinine, ratios [86]. However, it is only very recently that ghrelin has been shown to act directly on bones and not only exert its function through growth hormone release. Ghrelin significantly increased osteoblast-like cell numbers and DNA synthesis in a dose-dependent manner. Furthermore, ghrelin increased the expression of osteoblast differentiation markers and calcium accumulation in the matrix. Finally, ghrelin increased bone mineral content of both normal rats and GH-deficient rats [87].

26.5.8 Antiproliferative/Proliferative Effect

GHS-R1a receptors have been detected in a number of tumor cells [77], but the effect of ghrelin on cell proliferation is very variable depending on the tumor. In vitro ghrelin inhibits cell proliferation of thyroid carcinoma cell lines expressing the ghrelin receptor [75]. Similarly, in breast cancer, ghrelin inhibits tumor cell proliferation [88]. A synthetic ghrelin receptor agonist (hexarelin) has been demonstrated to inhibit growth of lung carcinoma cell lines [89]. In contrast in one prostate cancer cell line, ghrelin has been demonstrated to increase proliferation [90] and ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness [91].

It could be speculated that ghrelin, due to the GH-secreting effect, may act through IGF-I and increase the growth of existing tumors [92]. However, although other GHS compounds increase IGF-I levels significantly [3], it has never been shown that ghrelin increases IGF-I activity [93]. Importantly, no tumor progression was noted in tumor-bearing animals treated with ghrelin [94].

26.6 CLINICAL PERSPECTIVE OF GHRELIN AGONISTS AND ANTAGONISTS

Both agonists and antagonists of the ghrelin receptor may have important clinical applications. From the beginning of the 1980s, GHS compounds have been developed by a large number of pharmaceutical companies in an effort to substitute very expensive GH treatments with oral active small molecules. Though the capacity to increase GH secretion may not be sufficiently high for ghrelin and GHS compounds to replace GH administration, they seem to have other important GH-independent functions that are beneficial for patients suffering from catabolic conditions. For example, they increase acute food intake and appetite even in patients suffering from cancer cachexia [95], a population where increase in mortality is associated with low nutritional status. Similarly, a large number of severe chronic pathological conditions are associated with anorexia and cachexia that ghrelin and ghrelinlike compounds may cure to some extent.

Since it was discovered that ghrelin is the only peripherally secreted hormone that increases food intake, an antagonist for the ghrelin receptor that decreases appetite and weight gain has been keenly sought after. Based on the observation that the ghrelin receptor may signal constitutively in the hypothalamic arcuate nucleus, inverse agonists seem to be the optimal pharmacological approach to silence ghrelin and the ghrelin receptor-induced activity.

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MECHANISMS CONTROLLING ADIPOSE TISSUE METABOLISM BY THE SYMPATHETIC NERVOUS SYSTEM: ANATOMICAL AND MOLECULAR ASPECTS

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27.1	Introduction	785
27.2	Cellular Aspects of Adipose Tissue: Cell Types and Depots	786
27.2.1	Molecular Features of White versus Brown Adipocytes	787
27.3	Sympathetic Nervous System Innervation of White Adipose Tissue	789
27.3.1	Neuroanatomical Studies: Retrograde Tracing	790
27.3.2	Neuroanatomical Studies: Anterograde Tract-Tracing of SNS to WAT	792
27.4	Role of SNS Innervation on Adipocyte Proliferative Capacity	797
27.5	Role of Glyceroneogenesis in Physiological Control of Lipolysis and Thermogenesis: An Underappreciated Story	799
27.6	Catecholamine Signaling Mechanisms in Adipose Tissue	801
27.7	Summary	804
	References	804

27.1 INTRODUCTION

The mechanism(s) responsible for the link between obesity and metabolic diseases, such as insulin resistance and diabetes, is not completely known, but it is likely to involve abnormalities in free fatty acid metabolism. In general, the rate of fatty acid release into the systemic circulation is greater in obese than in lean persons [1]. In addition, an ever-increasing array of adipose-tissue-derived cytokines and endocrine

factors has been identified with suspected actions on insulin sensitivity and the cardiovascular system. In contrast, extreme deficiencies in body fat, such as lipodystrophy, also cause metabolic abnormalities, including insulin resistance and diabetes [2]. Therefore, it is important to understand the development and metabolic functions of adipose tissue and how these processes are regulated.

Because of the persistent demand for energy by tissues, especially the central nervous system (CNS), where energy status itself is monitored, human and nonhuman animals are challenged many times daily with the need to meet these energy requirements. This is not a trivial accomplishment, especially for small mammals that have higher metabolic rates than their larger counterparts and that have the added liability of a high surface-to-volume ratio resulting in increased heat loss [3]. Mismatches in the supply and demand of energy from ingested food results initially in the liberation of glucose from the almost trivial carbohydrate stores of glycogen [4]. Quickly the focus of readily available and utilizable energy switches to lipid, stored primarily in the form of triglycerides in white adipose tissue (WAT), through the process of lipolysis.

27.2 CELLULAR ASPECTS OF ADIPOSE TISSUE: CELL TYPES AND DEPOTS

Adipose tissue is classified into two major types: WAT and brown adipose tissue (BAT). This is based on gross appearance, cell-type-specific gene expression, and predominant type of adipocyte. WAT is named for its white/yellowish color. It contains adipocytes with a single large lipid inclusion (termed *unilocular*), resulting in displacement of the cytoplasm and the nucleus into a thin rim surrounding the lipid droplet. However, it is important to appreciate that even among WAT depots there can be significant differences in metabolic and hormonal sensitivity [5], and some of these depots actually contain a mixture of white and brown adipocytes (e.g. [6]).

BAT is named for its “brown” color, which is derived from its rich blood supply and an enormous number of mitochondria per cell [7], both of which contain iron–sulfurcluster proteins and hemes. In overall appearance the adipocytes in BAT are smaller than white adipocytes and are characterized by numerous small lipid inclusions (this is termed *multilocular*) (see Fig. 27.1). Although long recognized anatomically as a special organ structure, it was in the early 1960s that the true function of BAT was realized, when it was proposed to be thermogenic [8, 9]. Since then, substantial work has shown that BAT is uniquely capable of responding to various environmental stimuli to generate heat from stored metabolic energy. In response to sympathetic nervous system (SNS) activation, BAT undergoes an orchestrated hyperplastic and hypertrophic expansion, increased blood flow, and recruitment of lipid and carbohydrate fuels for oxidative metabolism [10–12]. A unique and critical element of this thermogenic mechanism for dissipation of the proton gradient in brown fat mitochondria was recognized to be due to a brown-fat-specific mitochondrial uncoupling protein (UCP) [13], which was also called *thermogenin* [14]. As discussed in greater detail by Ricquier and Bouillaud [15], this mitochondrial protein, now termed UCP1, allows controlled proton leakage across the mitochondrial inner membrane that generates heat at the expense of respiration-coupled adenosine triphosphate (ATP) production. This uncoupling activity in

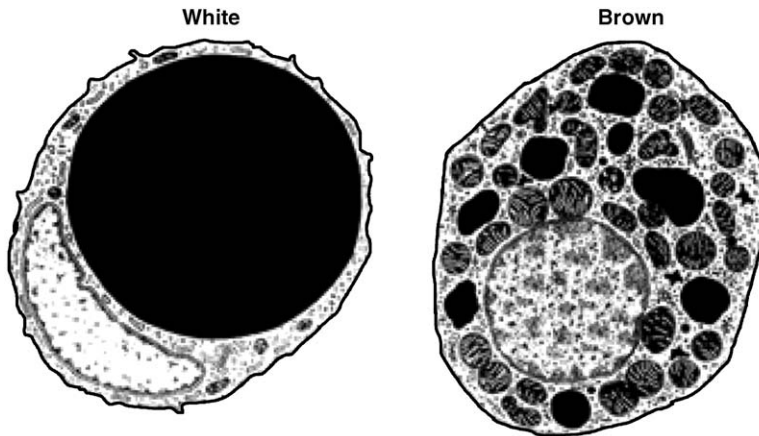


Figure 27.1 Sketch of morphological and structural distinctions between white and brown adipocytes. (Adapted with permission from [17].)

brown fat mitochondria is “activated” by free fatty acids that are released as a result of catecholamine-stimulated lipolysis of stored triglycerides.

BAT is most abundant in newborn mammals and is principally involved in heat production [7, 16]. In newborn humans, major BAT depots are located in the dorsal cervical, axillary, supriliac, and perirenal regions. With increasing age and size, bona fide homogeneous BAT depots disappear. This is also true for other large mammals such as dogs and nonhuman primates. However, brown adipocytes can also be found within white fat depots in the adult of all these species, particularly in the intra-abdominal and intrathoracic areas [17], but are relatively rarely in the subcutaneous depot. In the common small laboratory animals, such as mice and rats, BAT is very prominent in the interscapular and axillary areas from birth through adulthood. In fact, in newborn rodents BAT is the only adipose tissue, and it is only with suckling of the lipidrich milk that WAT depots begin to appear. In addition, brown adipocytes are present within WAT depots, such as the perigonadal, perirenal, and mesenteric fat, of adult rodents [18, 19]. Cold challenge or treatment with certain thermogenic agents further provokes the elaboration of brown adipocyte laboratory animals (Fig. 27.2); patients with pheochromocytoma possess large amounts of BAT [20] due to chronic catecholamine stimulation.

27.2.1 Molecular Features of White versus Brown Adipocytes

Despite the wealth of knowledge that has accrued over the past 25 years about the molecular events that set in motion and maintain the differentiated adipocyte phenotype, our understanding of the genetic programs that distinguish white from brown adipocytes is still far from complete. Historically, both the existence of brown adipose tissue in humans and the importance of brown adipocyte thermogenesis have had a very checkered past. It is clear that a discrete adipose depot of homogeneous brown adipocytes exists at birth but does not remain in adult humans. Nevertheless, one can also readily find “brown adipocytes” in adults, as defined by morphological and UCP1 histochemical criteria, scattered among white adipocytes in various

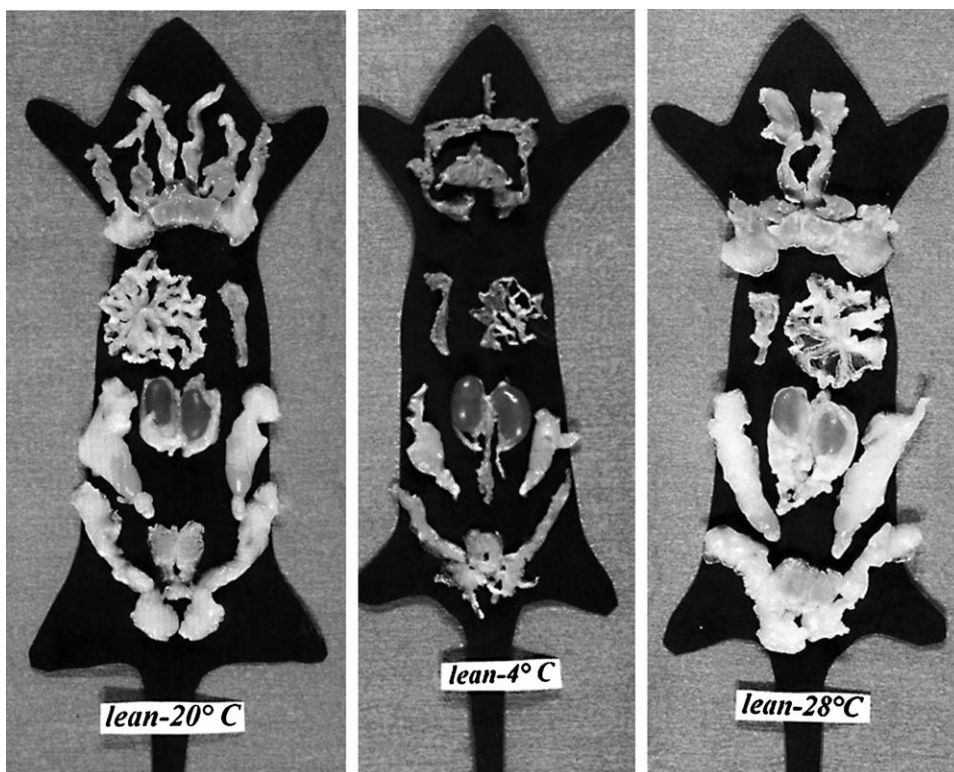


Figure 27.2 Dynamic environmental modulation of adipose structure and function. Note the striking changes in the cellularity and mass of adipose depots in response to environmental temperature adaptations. (Adapted with permission from [17].) (See color insert.)

“white adipose” depots, including perigonadal, perirenal, and pericardial (among others); albeit they appear to be a small percentage of total adipocytes. Environmentally, in response to cold exposure or overfeeding, mammals exhibit a complex response marked by increases in oxygen consumption, food intake, and heat generation through both shivering in skeletal muscle and nonshivering thermogenesis in BAT. At the cellular level, this “recruitment” of brown fat for thermogenesis occurs in response to noradrenergic stimulation and includes brown adipocyte proliferation, increased expression and activity of the brown-adipocyte-specific UCP1, and mitochondrial biogenesis [21]. All of these responses are mediated by the β -adrenergic receptors (β ARs). The mechanism(s) responsible for the proliferation of brown adipocytes is not understood, except to the extent that it can be mimicked by cyclic adenosine monophosphate (cAMP) analogues or the adenylyl cyclase activator forskolin [12]. There is also evidence that mitogen-activated protein (MAP) kinase cascades might be involved as there is evidence for growth promotion and protection from apoptosis in fetal brown adipocytes [22]. This may involve a combination of growth factor and direct β AR and cAMP stimulation of MAP kinases [23]. Thus, there are several important unanswered questions pertaining to the issue of brown adipocytes in adult humans. First, at the present time we have essentially no information about the median numbers of brown adipocytes in

adult humans. This is partly due to the fact that in the past most adipose tissue biopsies analyzed were subcutaneous and it was concluded that there are no brown adipocytes in adults. But the subcutaneous compartment is not a location of brown adipocytes at birth. Similarly, in small rodents that retain bona fide BAT depots in adulthood, one finds scattered brown adipocytes in intra-abdominal and intrathoracic white adipose depots but rarely in subcutaneous fat (except inguinal fat in certain mice is genetically linked to significant energy expenditure; reviewed in [18]). Second, even after we gain an accurate assessment of the numbers and locations of these scattered brown adipocytes in adult humans, we still must assess whether they are thermogenically active. Finally we must determine whether these cells can be recruited in greater numbers in response to pharmacological agents that behave as “thermogenic” drugs in rodent models. The unique morphological differences between brown and white adipocytes are the result of differential gene expression during their development and reflect the opposing metabolic functions of these cells: storage of caloric energy versus oxidation of caloric energy as heat. One of the most interesting questions remaining to be answered in the field of adipocyte development is how and what molecular “decisions” are made in the mesenchymal precursor cells that give rise to white versus brown adipocytes. Most evidence points to an important developmental and homeostatic role of catecholamines and the SNS.

27.3 SYMPATHETIC NERVOUS SYSTEM INNERVATION OF WHITE ADIPOSE TISSUE

Physiological dogma states that adrenal medullary catecholamines, especially epinephrine (EPI), are released into the circulation to trigger lipid mobilization from WAT adipocytes [24–26]. Although the role of EPI in lipid mobilization cannot be entirely dismissed (e.g., [27]), it now appears that (SNS) innervation of WAT is the principal initiator of lipolysis. It should be noted that while genetic manipulations have shown that lipolysis still can occur *in the absence of* β -adrenoceptors (i.e., so-called β -less mice [28, 29], the significance of this non- β -adrenoceptor mediated lipolysis under physiological conditions is unclear, especially since major developmental adaptations to this extreme molecular maneuver are unknown. Therefore, we will focus on the considerable, although far from complete, data supporting the role of sympathetic nerve-mediated lipolysis via β -adrenoceptors here.

There is a long history suggesting that WAT is innervated, beginning with the observation of the German anatomist Dogiel [30], who in 1898 simply showed ink-stained nerves entering WAT. Despite more than 100 years since this discovery, incontrovertible evidence of the SNS innervation of WAT only has developed recently with the advent of modern neuroanatomical techniques. These include methods that allow tracing of the SNS innervation from the site of the postganglionic neurons in the sympathetic chain to WAT [31] and, most recently, the use of transneuronal tract tracers revealing the CNS origins of the sympathetic outflow from brain to WAT [32]. A more detailed historical perspective laying the groundwork for the foundation upon which the notion that the SNS innervates WAT was built can be found in other recent reviews on the subject [33–35]. Some of these findings will be reviewed here, along with more recent developments in this area. There are three major functions of the SNS innervation of WAT: (1) lipolysis,

(2) control of fat cell proliferation, and (3) modulation of the synthesis/secretion of non-lipolysis-related substances that serve as autocrine, paracrine, and humoral signals. The SNS innervation of WAT will first be described in some detail, followed by the consequences of this innervation for lipolysis as well as for the control of fat-cell proliferation. The modulation of adipocyte-derived factors by the SNS innervation of WAT has been reviewed with regard to their possible effects on fat-cell proliferation [36] and in another chapter in this volume with respect to the control of leptin secretion by WAT (Chapter 25).

27.3.1 Neuroanatomical Studies: Retrograde Tracing

From the time of Dogiel, until ~30 years ago, progress in determining if WAT had SNS innervation and the extent (density) and type of innervation (vascular, parenchymal) was hampered by one of the defining characteristics of white adipocytes — the solitary lipid droplet that forces intracellular organelles, including the nucleus to the cell perimeter. This situation causes such tight packing of adipocytes in the fat-pad support matrix that the parenchymal space is virtually nonexistent with perivascular areas most prominent [17]. It is not surprising, therefore, that early reports described only vascular innervation, especially the vascular adventitia of the inter- and intralobular arteries and veins (e.g., [4, 37]. Indeed, in our own recent experience (e.g., [38–40] and the experience of others [17, 41, 42], this is what is most frequently, but not solely, observed at the light microscopic level of WAT from *ad libitum*-fed rodents. Despite this difficulty, electron microscopy reveals both direct and *en passant* sympathetic innervation of WAT [43–45]. When histofluorescence was applied to the problem, the nerves were unambiguously identified as catecholaminergic and, therefore, noradrenergic of sympathetic origin (e.g., [46–50]. The combination of histofluorescence analysis of tissues in the context of the fasted animal results in a reduced adipocyte size and thereby reveals some of the parenchymal space as well as increasing the catecholaminergic content of the nerves, making the nonvascular innervation more apparent [44]. Taking this one step further, under such fasting conditions combined with electron microscopy, indisputable evidence of nerve–adipocyte contact is observable [44]. Specifically, arteries and arterioles are encapsulated by catecholaminergic neurons and the innervation is most extensive for the mesenteric WAT and epididymal WAT (EWAT) pads, but only ~2–3% of the adipocytes receive direct innervation [44]. Using histofluorescence with confocal microscopy, a three-dimensional representation of the catecholaminergic innervation of WAT is achieved [51], but these results were presented only as a vivid narrative, without images.

A nonhistofluorescence approach to identify the SNS innervation of WAT is to use immunohistochemistry and stain for markers of sympathetic innervation. Tyrosine hydroxylase (TH), the enzyme that is rate-limiting for catecholamine neurons and acknowledged as a peripheral marker of noradrenergic nerves, is found in WAT nerves making contact with the vasculature and in the parenchyma [17, 40, 41, 52] (see Fig. 27.3). Although norepinephrine (NE) is the predominant postganglionic neurotransmitter of sympathetic neurons, including those innervating WAT, neuropeptide Y (NPY) also is colocalized with NE in most postganglionic sympathetic nerves (e.g., [53, 54]. For example, in subcutaneous, mesenteric, and perirenal WAT of the domestic pig, postganglionic sympathetic chain neurons labeled by application

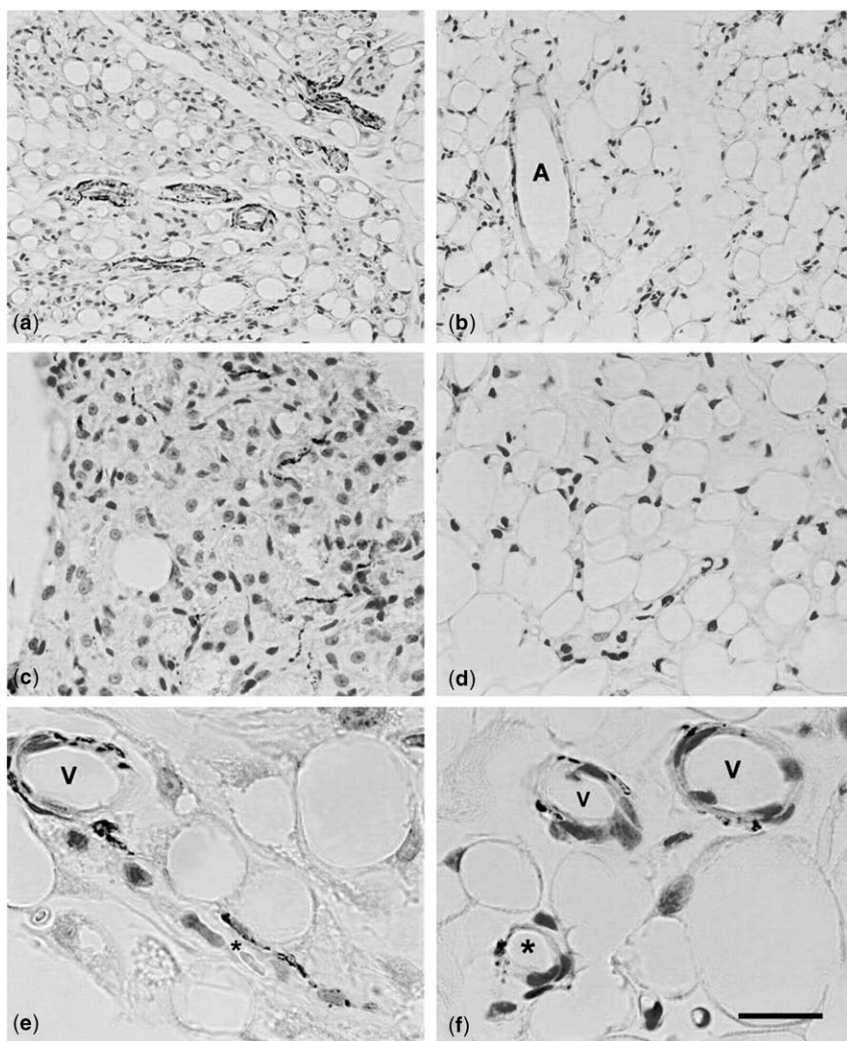


Figure 27.3 Sympathetic noradrenergic nerve distribution in retroperitoneal (a, c, e) and epididymal (b, d, f) WAT of rats that have lost 25% of body weight. Numerous noradrenergic nerves are present around intralobular arteries in the massively lipid depleted retroperitoneal WAT (a). A smaller number of noradrenergic nerves are found around arteries (A) in the less slimmed epididymal depot (b). As evident in highly delipidated adipose areas, the density of parenchymal noradrenergic nerves is greater in retroperitoneal (c) than epididymal (d) WAT. At high magnification, parenchymal noradrenergic nerves are seen around small blood vessels (V) and in close association with capillaries (asterisk) in both retroperitoneal (e) and epididymal (f) depots. Bar: a, b = 80 μ m; c, d = 40 μ m; e, f = 20 μ m. (Adapted with permission from [42].) (See color insert.)

of the retrograde tract tracer fast blue also contain NPY-immunoreactivity (ir) [55]. NPY-ir also is colocalized with TH-ir in the vast majority of perivascular and with some parenchymal nerves in laboratory rat EWAT and retroperitoneal WAT (RWAT) [42].

There is one report of parasympathetic nervous system (PSNS) innervation of WAT [56]. This conclusion is largely based on the use of a viral transneuronal tract tracer, the pseudorabies virus (PRV; see below for a more thorough discussion of this technique) to label PSNS outflow circuits to intra-abdominal fat pads in laboratory rats [56]. To visualize this PSNS innervation, these researchers contend that they remarkably were able to surgically denervate only the sympathetic innervation of WAT, sparing the posited PSNS innervation [56]. PRV was then injected into the denervated fat, resulting in PRV-infected neurons in the dorsal vagal complex of the brain stem, a classically defined PSNS region (e.g., [57]). Besides the difficulty, if not impossibility, of selective surgical denervation of the sympathetic *but not* the parasympathetic innervation (because of the identical outward appearance of sympathetic, parasympathetic, and myelinated sensory nerves), there is a likely alternative explanation for the PRV-labeled dorsal vagal complex neurons. That is, the notion that any brain area is solely sympathetic or parasympathetic has not withstood the test of time (e.g., [58, 59]). Thus, in the first report of the CNS outflow from brain to WAT as revealed by PRV injections into WAT [32], we also observed a few PRV-labeled neurons in the dorsal vagal complex but attributed them to rogue SNS outflow neurons rather than neurons of the PSNS. More selective tests of possible PSNS innervation of WAT, using 6-hydroxydopamine (6OHDA) to selectively destroy catecholaminergic neurons (i.e., SNS neurons) in WAT followed by injection of PRV to infect any of the remaining innervation, did not find evidence of WAT PSNS innervation [38]. This supports the lack of biochemical evidence [47, 60] for parasympathetic innervation. From these studies and attempts using other methods [61–64], we can find little or no neuroanatomical evidence for this innervation. Such PSNS innervation would be intriguing, however, in that it would afford WAT the fine neural control possessed by most other organs.

27.3.2 Neuroanatomical Studies: Anterograde Tract-Tracing of SNS to WAT

The first *direct neuroanatomical* evidence of the innervation of WAT in any species was accomplished by labeling Siberian hamster and laboratory rat postganglionic SNS neurons using both fluorescent anterograde and retrograde neuronal tract tracers injected into the sympathetic chain and in inguinal WAT (IWAT) or EWAT, respectively [31]. There were relatively separate populations of sympathetic chain postganglionic neurons innervating each adipose depot, with the EWAT pad having a more rostral pattern of labeling in the sympathetic chain compared with the IWAT pad [31]. Collectively, these results support and extend the histofluorescence evidence of SNS innervation of WAT and perhaps offer a neuroanatomical basis for the differential lipid mobilization seen by WAT pads across a variety of conditions, including age and anatomical location of the pads [65], exposure to short photoperiods [66–69], estrogen status [70], and food deprivation [70], among other manipulations.

As indicated above, the PRV can and has been used to define complete neural circuits within the same animal (for review see [71]). Before PRV, circuits were defined with standard tract tracers (e.g., DiI, FluoroGold, cholera toxin B subunit, rhodamine-labeled microspheres, biotinylated dextran amines) by labeling one connection within a circuit at a time between sets of animals. The process was rather laborious as it would be continued with additional sets of animals given tracer injections into either

the end (retrograde) or beginning (anterograde) of the single-circuit component that had been defined in the prior set of animals. Interpretations had to be cautious since the sequential labeling of neurons within a circuit across animals cannot be done in a manner that guarantees that the injection of the tract tracer in the subsequent set of animals will be at the *precise location* of the next neurons in the chain comprising the circuit. This problem was alleviated with PRV because, once injected in the suspected target of the projecting circuit, it only travels in a retrograde fashion and only progresses from neuron to neuron if the cells are synaptically connected, thus yielding a hierarchical chain of functionally connected neurons labeled from the end of the circuit to its beginnings. Because the virus is replicated in each of the neurons in the circuit, an equivalent intensity labeling (i.e., “nondampening”) of each neuron occurs (for review see [71]). SNS outflow circuits have been labeled from brain to EWAT, RWAT, IWAT, and interscapular BAT (IBAT) in Siberian hamsters [32, 72–76] and IWAT and EWAT in laboratory rats [32]. Figure 27.4 shows an example of discretely labeled neuronal regions following PRV injection into IBAT [72]. Superficially, there

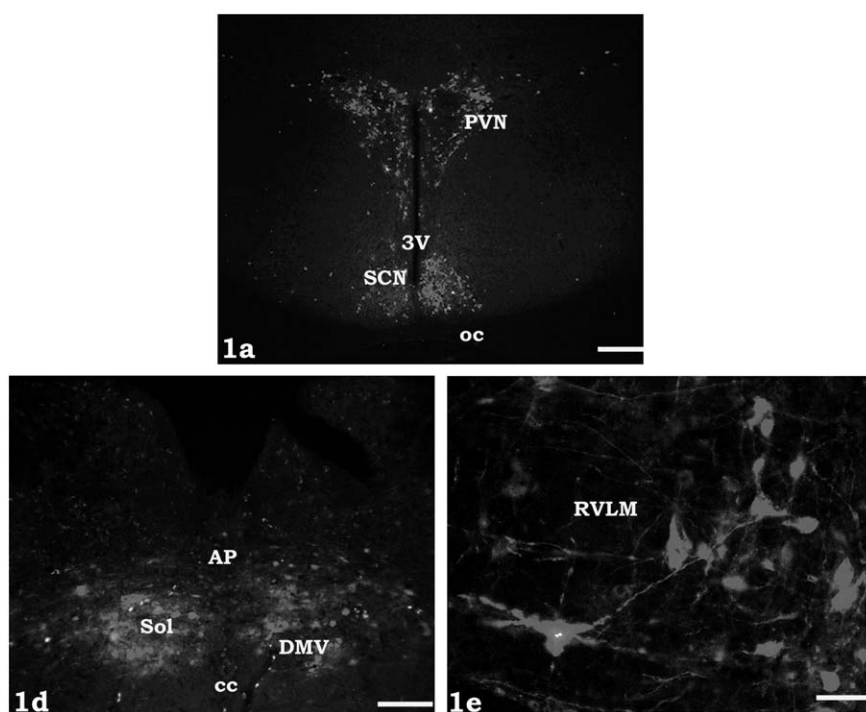


Figure 27.4 Photomicrographs illustrating distribution of PRV-labeled cells in cross sections of brain from a representative Siberian hamster six days after PRV that has a green fluorescent protein (GFP) reporter was injected into IWAT. *Top:* Hypothalamic section at level of suprachiasmatic nucleus and paraventricular nucleus. *Bottom left:* Section in caudal brain stem at level of dorsal vagal complex. *Bottom right:* Section at level of medulla brain stem showing the rostroventrolateral medulla. Abbreviations: 3 V, third ventricle; AP, area postrema; cc, central canal; DMV, dorsal motor nucleus of vagus; oc, optic chiasm; PVN, paraventricular hypothalamic nucleus; RVLM, rostroventrolateral medulla; SCN, suprachiasmatic nucleus; Sol, nucleus of solitary tract. Each scale bar represents 100 μm . (Adapted with permission from [73].) (See color insert.)

are more similarities than differences in the labeled SNS outflow circuits among WAT pads and between species. One exception is in the spinal cord and sympathetic chain, where a more easily discernable viscerotopic rostral-caudal distribution of sympathetic neurons occurs with more rostrally located pads showing infected neurons in more rostral segments of the sympathetic chain and spinal cord. Common areas showing some degree of infection after PRV injection across the WAT pads and between the two species are the spinal cord (IML cell group, central autonomic nucleus), the brain stem (nucleus of the solitary tract, area postrema, lateral paragigantocellular nucleus), raphe regions (raphe pallidus, raphe obscurus, raphe magnus), and reticular regions, especially the parvicellular lateral reticular nucleus and rostroventrolateral reticular nucleus/adrenaline cells (C1, A5 regions), midbrain (periaqueductal gray, mostly in the lateral periaqueductal gray and pedunculopontine tegmental nuclei, dorsal raphe), and forebrain (arcuate nucleus, dorsal and lateral hypothalamic areas and paraventricular nuclei, suprachiasmatic and dorsomedial nuclei, medial preoptic area, zona incerta, medial amygdala, septum, and bed nucleus of the stria terminalis) [32, 73–76].

A complete description of the neurochemical phenotype of some of the PRV-labeled SNS outflow neurons from brain to WAT is in its infancy, but some phenotypic information is known. First, as noted above, many postganglionic neurons are both catecholaminergic and contain NPY [42]. In the initial neurochemical survey of the PRV-labeled SNS outflow neurons in Siberian hamster brain, we tested for PRV colocalized with TH, dopamine- β -hydroxylase (DBH), phenylethanolamine-*N*-methyltransferase, arginine vasopressin (AVP), oxytocin (OXY), somatostatin, substance P, NPY, corticotropin-releasing hormone (CRH), and hypocretin [74]. There were some double-labeled catecholaminergic neurons (TH- and DBH-ir) and some peptidergic neurons (AVP- and OXY-ir) in this outflow, but there was a low percentage of double-labeled cells overall, consistent with other PRV studies that attempted to chemically code the SNS projections from the brain to peripheral tissues (for review of comparison labeling see [74]). At least a partial reason for the generally low levels of colocalization is likely a result of virus-induced inhibition of peptide synthesis [71]. The brain stem TH + PRV and DBH + PRV cells were in previously identified populations of noradrenergic neurons and were especially prominent in the A5, A6, and subceruleus areas as well as the rostroventrolateral medulla and some reticular nuclei. The most impressive forebrain doublelabeling was in the hypothalamic PVN, where PRV + AVP and PRV + OXY cells predominated but there also was some PRV + TH colocalization [74].

Because the PRV methodology can be combined with in situ hybridization, this is an especially effective means of identifying receptors possessed by PRV-infected cells because receptor antibodies for immunohistochemical analyses often do not exist, are not specific, or have insufficient sensitivity to label receptors found in low concentrations. For example, we have been studying the reversal of a naturally occurring seasonal obesity in Siberian hamsters (for reviews see [77–79]). The environmental cue that triggers lipid mobilization in these animals is the rate of change of the photoperiod (day length); this is transduced into a neuroendocrinological signal through the duration of the nocturnal secretion of melatonin (MEL; for review see [80]) such that in short “winterlike” days (long nocturnal duration of MEL secretion) body fat decreases [27, 31, 66–68]. Photoperiodic responses, including the changes in body fat, are therefore triggered by these photoperiod-encoded MEL signals. MEL

does not directly trigger lipid mobilization (lipolysis), as shown by *in vitro* additions of MEL to isolated adipocytes [82]. In addition, none of the hormones that change seasonally that also have been shown to affect lipolysis in other species either directly or indirectly (e.g., gonadal steroids, prolactin) could account for the short-day-induced increases in lipid mobilization (for review see [77]). These data coupled with the inability of adrenal demedullation to block these short-day-induced increases in lipid mobilization [27] suggested that MEL interacts with the sympathetic nerves to mediate this response. Therefore, we labeled the SNS outflow to WAT by injecting PRV into IWAT of Siberian hamsters and tested for colocalization of mRNA for the functional MEL receptor responsible for photoperiodic responses (MEL_{1a} receptor subtype) [75]. There were PRV-ir cells that also had MEL_{1a} receptor messenger ribonucleic acid (mRNA) in the suprachiasmatic nucleus (SCN), perifornical area, periventricular fiber system, hypothalamic paraventricular nucleus (PVN), zona incerta (ZI), anterior hypothalamic (AH) nucleus, dorsomedial nucleus (DMN), thalamic paraventricular nucleus, and reuniens/xiphoid nuclei, among other areas [75]. Using a combination of neuroanatomical data with a variety of other measures [27, 31, 69, 83], the findings demonstrate that the SNS innervation of WAT mediates the short-day reversal of seasonal obesity in these animals.

The role of the SNS innervation of WAT is not confined to short-photoperiod/MEL-induced increases in lipid mobilization (see below) and accordingly involves other neurochemical systems. For example, the melanocortins have been implicated in the control of energy balance (for review see [84]). The melanocortin-4 receptor (MC4-R) likely is the key receptor subtype mediating these responses. Neurons in the brain synthesize and release both an agonist (α -melanocyte-stimulating hormone [α -MSH], a cleaved product of the polypeptide precursor pro-opiomelanocortin), and an antagonist (agouti-related protein) to MCR-Rs. Central melanotan II administration, a synthetic analogue of α -MSH [85], decreases WAT pad mass more than can be accounted for by its ability to decrease food intake [86], suggesting increases in mobilization of lipid that could be mediated by the sympathetic innervation of WAT. In support of this, the same maneuver increased norepinephrine turnover (NETO) in WAT of Siberian hamsters (Brito, N., Brito, M., and Bartness, T., in preparation). Separately, MC4-R mRNA was found by *in situ* hybridization to be highly ($\sim > 60\%$) colocalized in PRV-labeled cells across the neuroaxis, including areas typically implicated in the control of lipid mobilization (e.g., hypothalamic PVN, SCN, DMN) [76]. These neuroanatomical data and the functional data cited above strongly suggest that the melanocortins play an important role in lipid mobilization through MC4-Rs to alter lipid mobilization and adiposity.

Differential responses of WAT pads *in vivo* or of isolated white adipocytes harvested from these pads clearly indicate that WAT as an organ shows variations in responses depending upon the anatomical location. If multiple fat pads are assayed, then almost regardless of the measure, at the very least the magnitude of the response in question tends to differ among the WAT depots. This concept has been elegantly presented from a developmental and anatomical perspective by Cinti [17]. One example we can discuss here is lipolysis [5]. There are numerous possible mechanisms underlying differential lipolytic rates among fat pads, including variations in blood flow, cellularity [fat-cell size (FCS) or fat-cell number (FCN)], as well as differences in the density of sympathetic innervation (number of nerve fibers) and including the expression of the various adrenoceptor subtypes (see Section 27.6). In

terms of the latter, a primary factor underlying differential WAT lipolytic rates is the ratio of β -adrenoceptors to α_2 -adrenoceptors (for reviews see [87–89]) and is described in detail below.

Beyond the scope of this review but worthy of consideration in another forum is the role of factors other than α_2 -adrenoceptors that inhibit lipolysis and could therefore counter increases in sympathetic drive or changes in the relative number/affinity of β -adrenoceptor subtypes to diminish lipid mobilization (adipocyte-derived adenosine, insulin). NPY is an additional antilipolytic factor that is a component of the SNS innervation of WAT. As noted above, WAT sympathetic nerves most often contain NPY as well as NE. NPY receptors, as measured by ^{125}I -labeled peptide YY (PYY) binding sites, are found on white adipocyte membranes isolated from a variety of species, including humans, dogs, and garden dormice, but not Syrian hamsters, laboratory rats, and guinea pigs [90]. Of those species, garden dormice and to a lesser degree dogs and humans show impressive antilipolytic responses to NPY alone [90], although others report an $\sim 25\%$ inhibition of lipolysis in laboratory rat white adipocytes in the presence of NPY, PYY, or $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ (the latter a NPY Y1 receptor subtype agonist), but not NPY^{13-36} , a NPY Y2 receptor subtype agonist [91]. The NPY receptor subtype involved in the antilipolytic response of NPY and PYY may be species specific, however, because dog adipocytes respond in the opposite manner— NPY^{13-36} , but not $[\text{Leu}^{31}, \text{Pro}^{34}]\text{NPY}$ causes antilipolysis [92]. The antilipolytic responses to NPY are most easily explained by the presence or absence of ^{125}I -labeled PYY binding sites white adipocyte membranes in these species [90]. Finally, although one usually conceptualizes the communication between the SNS and fat as unidirectional via postganglionic secretion of NE and/or NPY, there is evidence of adipocytes affecting the postganglionic sympathetic nerves [93]. In addition, the existence of a variety of adipose-derived peptides such as leptin, adiponectin, and resistin may have local as well as distal effects on adipose tissue metabolism through their effects on other organs including the CNS.

Differential lipolytic rates among WAT pads occur in a wide variety of stimuli in vivo, although the exact mechanisms and contributions of the assorted factors underlying the various degrees of lipid mobilization are not precisely known. For example, the reversal of the seasonal obesity associated with transfer from long “summer like” to short winterlike days in Siberian hamsters, discussed above, is associated with a more rapid and pronounced lipid mobilization (reduction in fat pad mass) [66–68] and lipolysis (glycerol release from isolated adipocytes) [69] in the more internally located RWAT and EWAT pads than in the more externally located subcutaneous IWAT pad. Because starvation increases WAT NETO [94] and lipid mobilization from WAT is blocked by surgical denervation [95–98], but not adrenal demedullation [99, 100], this differential effect is reasonably concluded to be mediated by the SNS innervation of these fat pads. It may be, however, that the exact sympathetic-related mechanisms underlying differential lipolytic rates occur through a suite of changes. For example, our finding of greater short-photoperiod-induced lipid mobilization from EWAT than IWAT [27, 66–69] could have occurred only via the greater short-photoperiod-induced NETO increases in the former [31]. We subsequently found, however, greater short-day-induced increases in β_3 -adrenoceptor mRNA (and likely β_3 -adrenoceptors at the cell surface) in EWAT than in IWAT [101] and similarly significant increases in NE-induced potency (sensitivity/effective concentration EC_{50}) and efficacy (maximal response asymptote) by short-day

exposure for both pads [69]. Thus, it appears as though the short-day-induced increases in sympathetic drive to WAT, as well as changes in the potency or efficacy of postganglionically released NE, and perhaps β_3 -adrenoceptor number primarily underlie the differential lipid mobilization of EWAT versus IWAT. Furthermore, these data suggest that multiple measures of sympathetic involvement are needed to determine the exact mechanisms underlying differential lipid mobilization rates seen *in vivo*.

Another factor that likely could affect lipid mobilization as well as other sympathetic responses in WAT is differences in sympathetic innervation. Differential SNS innervation densities across WAT depots was first suggested in a written description of the histofluorescent identification of WAT catecholaminergic innervation when it was combined with confocal microscopy three-dimensional images [51], as noted above. These descriptions of direct catecholaminergic innervation of adipocytes and vasculature in laboratory rat WAT paint a written picture of the differences in the density of this innervation among several WAT pads. More specifically, IWAT was the least and mesenteric WAT the most densely innervated (i.e., mesenteric > EWAT > RWAT > IWAT [51]). This pattern of innervation mirrors the highest and lowest rates of NE-stimulated lipolysis from isolated adipocytes harvested from these pads (e.g., [102]). More recently, using standard immunohistochemistry for TH, the sympathetic nerve marker, and PGP9.5, the pan-peripheral nerve marker, a different order of innervation density is suggested in laboratory rats, with RWAT more densely sympathetically innervated than EWAT [42]. Accordingly, fasting decreases RWAT mass more rapidly and to a greater extent than EWAT within the same animal [42]. Moreover, fasting stimulates increased innervation of RWAT, but not EWAT, as evidenced by increases in PGP9.5-ir that is colocalized with TH-ir nerves, the latter suggesting that increases in sympathetic innervation also could contribute to the magnitude and more rapid time course of fasting-induced lipid mobilization RWAT compared with EWAT [42]. Not to be neglected in this context is the fact that RWAT depots in many species typically contain a mixture of white and brown adipocytes. Therefore, this depot is capable of dissipating its triglyceride stores *in situ* in addition to mobilizing the fuel for export.

27.4 ROLE OF SNS INNERVATION ON ADIPOCYTE PROLIFERATIVE CAPACITY

Although the primary focus here has been the sympathetic neural control of lipid mobilization, there is another, slower to be recognized function of this innervation—the control of adipocyte proliferation. Hypercellularity is a hallmark of obesity [103] and one of the important unsolved mysteries of adiposity. Understanding factors controlling adipocyte proliferation are important because if FCN did not increase, there would be severe limits to the degree of fatness that could be reached because maximum obesity would result with lipid filling of the existing adipocytes to capacity. Although there is a host of circulating and paracrine factors that affect proliferation (for review see [36]), there is growing support for the role of the SNS innervation of WAT playing a highly significant part in this process. The first clear evidence of this role was the ability of NE to inhibit preadipocyte proliferation *in vitro* [104]. Specifically, the normal proliferation of a primary culture of rat white adipocyte

precursor cells is inhibited by incubation with physiological concentrations of NE [104]. Moreover, pretreatment of these cultures with the pan β -adrenoceptor antagonist propranolol blocks the ability of NE to inhibit proliferation [104]. These results suggest that white adipocyte precursor cells have functional β -adrenoceptors, most likely β_2 AR, which is expressed at low levels in essentially all cell types including mouse preadipocytes. Consistent with this notion, β -adrenoceptors have been identified on adipocyte precursor cells [105]. The inhibition of fat-cell proliferation by NE does not appear to be an *in vitro* curiosity because local surgical denervation of WAT *in vivo* stimulates white adipocyte proliferation in laboratory rats [106] and Siberian hamsters [40, 73, 83]. Because many WAT pads are bilaterally located and unilaterally innervated, experiments can be done where one side is surgically or chemically denervated with its corresponding contralateral mate receiving sham surgery or vehicle injections and thus acting as a within-animal control. Therefore, within an animal, its age, genetics, nutritional status, and circulating factors are the same for both fat pads, but one is denervated and the other intact. Unilaterally denervated Siberian hamster IWAT doubles its mass compared with the contralateral sham denervated pad, an effect exclusively due to a mirrored doubling of FCN with little change in FCS [73, 83]. Because obesity typically is associated with decreases in sympathetic drive (for reviews see [107–109]) and increases in FCN in rodent obesity models [110–113] and in humans [114, 115], it seems likely that a primary influence on obesity-associated increases in white adipocyte proliferation is decreases in SNS activity.

The above data suggest that the lack of sympathetic innervation triggers increases in FCN. This conclusion is based on the assumptions that (1) the key feature of the surgical denervation is interruption of the SNS innervation of WAT and (2) the increases in FCN are based on increases in fat-cell proliferation rather than increases in differentiation of immature adipocytes into mature ones. In terms of the first point, WAT possesses both sympathetic as well as sensory innervation but not parasympathetic innervation (see above); therefore, surgical denervation would nonselectively sever both sympathetic and sensory nerves. By a variety of approaches [40, 116, 117] the results collectively conclude that sympathetic denervation, not sensory denervation, triggers adipocyte precursor cell division resulting in increases in FCN.

The magnitude of the SNS denervation-induced increase in fat-cell proliferation appears to reflect the propensity of the WAT pad—the inherent genetic constitution of the adipocyte itself—to naturally show increases in adipocyte proliferation. For example, surgically denervated Siberian hamster IWAT achieves a greater percent increase in FCN than similarly treated RWAT [73], whereas EWAT surgical denervation does not affect FCN [40]. This corresponds nicely to the predisposition of EWAT to increase its mass by increasing FCS, whereas RWAT and IWAT do so primarily by increasing FCN in the obesity associated with aging in Wistar rats [118]. In addition, the *in vivo* incorporation of radiolabeled thymidine into DNA in RWAT (a radioactive method of identifying dividing cells) is greater than that of EWAT in high-fat-diet-fed rats [119], further supporting the tendency of the former to expand primarily by increases in FCN. Therefore, it may be that the inhibition of sympathetic drive associated with obesity or produced experimentally by surgical or chemical denervation promotes increases in adiposity not only by decreasing basal and stimulated lipolysis but also by increasing fat-cell proliferation. There is also an interesting reciprocal relation between denervation and the expression of the

β -adrenergic receptors such that the denervated fat pad exhibits significantly greater mRNA levels for β_1 AR and β_3 AR, reflecting a tonic SNS feedback influence on receptor density [120]. This has been observed for β ARs in other situations such as in the heart following chronic β -blocker therapy [121] or even in IBAT of obese animals [122]. Finally, the cellularity changes underlying the increases in fat mass may be reflected in the programmed changes in cellularity of the various fat depots. This latter aspect tends to be underappreciated, but there is ample evidence in the literature from early studies of the mouse obesity mutants such as *ob/ob* (Lep^{ob}/Lep^{ob}) and *db/db* ($LepR^{db}/LepR^{db}$) [123–125] that clearly indicates that the phenotypes of these mutants are significantly dependent upon the strain background on which they are bred. This early work has been extended by others investigating the marked strain-dependent responses of nonmutant mice to a dietary obesity challenge [37, 126, 127], including marked strain differences in pancreatic insulin secretion [128], adaptations to the diet by changes in fat cell size versus number [127], and relative contribution of physical activity [129], among others. With the popular generation of transgenic and, more importantly, targeted “knockout” mutations that are generated in hybrid strain backgrounds of the 129/Sv substrains [130] crossed with C57BL/6J, there has been a resurgence of this interest in the effects of strain background on the outcomes of studies focused on diabetes and obesity phenotypes [131].

27.5 ROLE OF GLYCERONEOGENESIS IN PHYSIOLOGICAL CONTROL OF LIPOLYSIS AND THERMOGENESIS: AN UNDERAPPRECIATED STORY

Weight homeostasis and maintenance of body energy stores involve the control of energy expenditure in addition to control of food intake. The SNS has an important role in the control of energy expenditure (adaptive thermogenesis) through its neurotransmitter, NE, acting upon α - and β -adrenoceptors to influence heat production. It has been known for many years that the two forms of thermogenesis (diet-induced thermogenesis [DIT] and thermoregulatory thermogenesis) can be mediated by BAT. The activity of BAT, which is abundant in small rodents and human infants, is directly controlled by the SNS. The activation of a BAT-specific mitochondrial protein (UCP1) by the SNS produces an uncoupling of substrate oxidation from phosphorylation and generation of ATP, with a resultant increase in heat production [10]. In human adults, however, the SNS control of energy expenditure is not mediated by discrete depots of bona fide BAT, which is scarce and without a clearly demonstrated function in adults. Although there is increasing recognition that brown adipocytes are retained into adulthood scattered within white adipose depots, their number and contribution to thermogenesis are purely a matter of speculation. Rather, the preponderance of interest has been on the potential activation of futile metabolic processes in other tissues. In which tissue(s) and by what molecular mechanisms the SNS acts to stimulate DIT remain unclear and are still the object of intense investigation and conjecture, particularly since animals lacking UCP1 actually seem to be leaner than their wild-type counterparts [132].

The maintenance of adequate stores of triglyceride is essential for a normal functioning of both BAT and WAT. The first step in the process of activation of BAT thermogenesis is the hydrolysis of endogenous triglyceride, the fatty acids (FAs) thus

produced being at the same time the main substrates for heat production and activators of UCP1, uncoupling oxidative phosphorylation [133]. Adequate stores of triglyceride also are needed for WAT to exert its basic function of mobilizing FA to attend to the demand of energy in different physiological conditions. The preservation of triglyceride reserves in the two tissues requires a continuous and controlled supply of glycerol-3-phosphate (G3P) to esterify newly synthesized or preformed FA (recycled after hydrolysis of endogenous triglyceride or taken up from the circulation, where they are incorporated into triglycerides of lipoproteins). For many years, only two sources of G3P for acylation and triglyceride formation were generally recognized: (1) glucose, via dihydroxyacetone in the glycolytic pathway, and conversion to G3P by glycerol-phosphate dehydrogenase, and (2) glycerol, produced by hydrolysis of stored triglyceride or taken up by the tissue from the circulation and phosphorylated to G3P by glycerokinase (GyK). In the case of WAT, which has low levels of GyK, phosphorylation of glycerol was considered negligible and glucose alone was considered to be the only significant source of G3P, thus making FA esterification to form triglyceride in WAT highly dependent on the utilization of the hexose. It was demonstrated almost 40 years ago that pyruvate and glucogenic amino acids also can be converted to glyceride-glycerol at appreciable rates in WAT [134–137]. The formation of TAG-glycerol was shown to proceed through a pathway that was named *glyceroneogenesis* [137], involving the carboxylation of pyruvate to oxaloacetate, decarboxylation of oxaloacetate to phosphoenolpyruvate, and subsequently production of G3P through a partial reversal of glycolysis. The key glyceroneogenic enzyme was determined to be cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) [134, 136]. This pathway remained practically ignored in the literature, its importance in the generation of G3P being fully recognized only recently (see [138]). The first clear in vivo evidence of the importance of glyceroneogenesis in lipid metabolism was the demonstration in nonanesthetized, freely moving rats that glyceroneogenesis is present not only in WAT but also in BAT and liver and that approximately 50% of G3P incorporated into WAT TAG is generated by this pathway [139]. Of great impact were the findings that the tissue-specific ablation of PEPCK-C gene transcription in mice produces an increased mobilization of FA and lipodystrophy [140] and that the overexpression of PEPCK-C results in reduced FA mobilization and obesity [141]. These studies definitively established PEPCK-C as the key enzyme of glyceroneogenesis. The finding that the reduced FA mobilization in rats treated with thiazolidinediones, an antidiabetogenic agent, is mainly due to an induction of adipose tissue PEPCK-C and increased glyceroneogenesis spurred the investigation into a potential role for altered transcriptional regulation of the PEPCK-C gene in subjects that are obese and/or type 2 diabetic [142].

Numerous studies, many recently reviewed [143], have been published on the molecular aspects of the induction of PEPCK-C expression, particularly on the regulatory regions of the promoter. In contrast, the regulation of the three G3P-generating pathways themselves (glycolysis, glycerol phosphorylation by GyK, and glyceroneogenesis) under different physiological conditions has been a topic of less frequent investigation. As for placing adipose tissue GyK in the context of thermogenesis, recent studies strongly suggest that BAT GyK activity is directly controlled by the SNS. For example, cold exposure results in a stimulation of BAT GyK activity that is completely blocked by tissue denervation [144]. Similarly, cold induction in GyK activity is paralleled by a marked increase in BAT GyK mRNA

levels that is inhibited by previous administration of propranolol or actinomycin D [144]. BAT GyK activity also is stimulated by chronic subcutaneous administration of NE or β -adrenoceptor agonists. However, the increase in enzyme activity is not immediate, requiring at least 12 h of cold exposure [144]. One possible explanation could be that the half-life of the enzyme protein is long, but this would need to be assessed directly. The activity of GyK in WAT is about 10 times lower than in BAT, but there are similar indications that WAT GyK also is controlled by the SNS. Unilateral surgical denervation of retroperitoneal WAT causes a reduction of GyK activity compared with its contralateral intact mate. In addition, GyK activity can be observed to increase markedly in WAT from cold-exposed rats. Thus, although further work is necessary to refine our understanding of the mechanisms controlling PEPCK-C transcription and enzymatic activity, it is clear that this glyceroneogenic pathway serves an important role in the control of WAT and BAT physiology.

27.6 CATECHOLAMINE SIGNALING MECHANISMS IN ADIPOSE TISSUE

Since we have now discussed the anatomical aspects of sympathetic innervation of adipose tissue and some of the functional differences between depots such as relative differences in catecholamine sensitivity between adipose depots for the mobilization of stored triglycerides, this section will focus on the signaling mechanisms emanating from the ARs to regulate lipolysis and thermogenesis. They are members of the large family of G-protein-coupled receptors that are integral membrane proteins of the plasma membrane. The ARs most important for the mobilization of stored fuel and promoting nonshivering thermogenesis are the β ARs. There are three subtypes of β ARs (β_1 AR, β_2 AR, and β_3 AR) [145–147], all of which are expressed in white and brown adipocytes [148–151]. However, the relative proportions of these subtypes vary between species, fat depots, and metabolic status [152]. The interest in the regulation and function of the adipocyte-specific β_3 AR as a potential therapeutic target for human obesity and metabolic disease has waxed and waned over the past 20 years. Initially it was considered an ideal target because of its selective tissue expression, along with pharmacological evidence that its activation could promote powerful thermogenic transformations of adipose depots from being composed largely of white adipocytes into a mixed white/brown adipocyte population. This was coupled with increased oxygen consumption and an overall leaner body composition. However, the relative expression of the β_3 AR in human adipose tissue is so much lower than in rodents as to be of questionable relevance. In earlier studies β_3 AR was found essentially only on brown adipocytes (reviewed in [18]). However, a number of studies are beginning to show lesser but nevertheless detectable amounts in human white adipocytes (importantly by functional criteria such as cAMP production in response to highly selective agonists). Two other issues bear further basic investigation before there would be any revival of interest in this receptor as a therapeutic target (these issues are discussed in [18]). First, chronic treatment with selective β_3 AR agonists, even in rodents, leads to a tachyphylaxis that is to some extent occurring at a point beyond the receptor itself ([122]; S. Collins, unpublished observations). The molecular features of this process must be understood. Second, studies examining human adipose tissue have invariably analyzed material that is either from subcutaneous depots—which is a location that contains few *if any* brown adipocytes under any

circumstance—or from intra-abdominal fat most often from obese individuals—also an unlikely source of these cells. Moreover, given the fact that these “brown adipocytes” that appear in animal models in response to β_3 AR agonist treatment might not be detected prior to treatment, there is also a question as to how “permanent” these changes are, for example, in the chronic rodent experiments referred to above. Therefore many issues surrounding not only the β_3 AR but the genesis and plasticity of brown adipocytes in adult humans still require significant investigation. In addition, efforts to mobilize stored triglyceride (TG) in white adipocytes are all well and good, but the FAs released must go somewhere to be oxidized.

The control of lipolysis by the β ARs in white and brown adipocytes is principally initiated by the sequential activation of adenylyl cyclase and cAMP-dependent protein kinase (PKA). These events ultimately culminate in the phosphorylation of various targets, including hormone-sensitive lipase (HSL) and perilipin A [153–157]. It is important to make the point that HSL has been ascribed through the years as the key necessary and sufficient lipase for TG hydrolysis and release from adipocytes into the circulation. But results from targeted disruptions of the HSL gene in mice gave irrefutable evidence that (an)other lipases existed, because relatively normal TG hydrolysis remained, with instead a net accumulation in tissues of diglycerides [158, 159]. In 2004 Zechner and colleagues reported the isolation of a new TG lipase that they termed ATGL (adipose triglyceride lipase) [160]. Although this lipase appears to be a rate-limiting step in cAMP-stimulated lipolysis, it is apparently not itself a phosphoprotein target of PKA. Some of these issues have been recently reviewed [161]. As briefly noted above, in addition to the β -adrenergic stimulation of lipolysis, catecholamines can also be antilipolytic themselves through their interaction with the α_2 ARs and its resulting inhibition of cAMP production. The balance between the relative amounts of the β AR and α_2 AR can thus determine the relative efficacy of catecholamines for TG hydrolysis. In that respect, there is evidence from experimental studies in animals and humans that a shift to a higher α_2/β ratio can contribute to net lipid storage [88]. The lipases and lipid droplet binding proteins that control TG hydrolysis are targets of the signaling cascades initiated by the adrenergic receptors [19, 162]. However, in addition to the newly discovered ATGL, there are likely to be additional as-yet-unidentified lipases and, particularly of interest, lipid droplet scaffolding proteins with regulatory roles. The latter include the newly discovered S3-12 protein [163] as well as a possible PKA-dependent chaperone or cofactor for the activation of ATGL.

The basic biochemical scheme for the stimulation of lipolysis is presented in Fig. 27.5. Also included in this cartoon is an expanded view of this process as a result of new developments in our understanding of the downstream signaling cascades triggered by β AR activation in adipocytes (reviewed in [18]). These include the activation of various MAP kinase (MAPK) cascades in addition to the well-established cAMP/PKA pathway. These include ERK1/2 MAPK [164] and p38 MAPK [165, 166]. They appear to be independent of each other and activated in adipocytes in response to catecholamines by different mechanisms, but this also depends upon the source of adipocytes. Activation of the ERK1/2 MAPKs by β -adrenergic agonists occurs as a result of receptor coupling to the heterotrimeric G protein Gi [164] and does not involve PKA [165], while p38 MAPK activation is downstream of β -agonist increases in cAMP levels and PKA activity [165, 167]. The

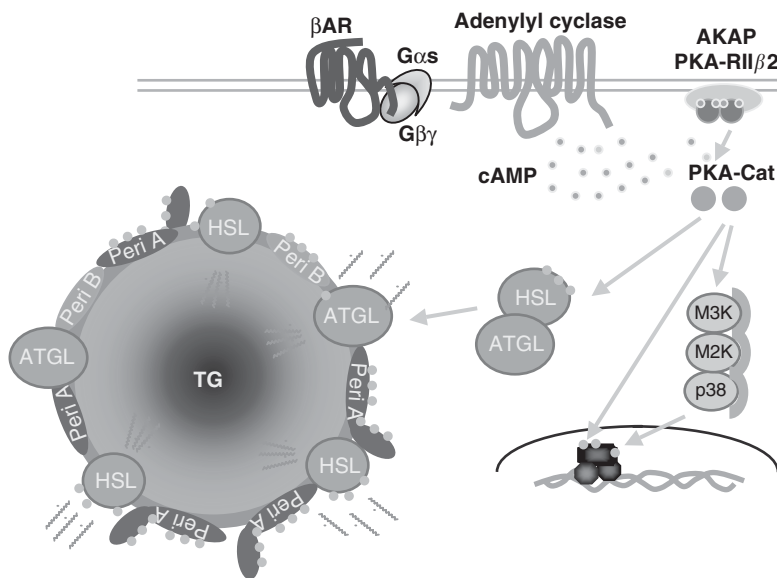


Figure 27.5 Simple cartoon depiction of events in β AR stimulation of adipocyte TG hydrolysis. When catecholamines interact with the β ARs, they couple through a heterotrimeric G protein to adenylyl cyclase. The cAMP produced binds to the regulatory subunits of PKA, which are bound to juxtamembranous anchor proteins called AKAPs. The released active catalytic subunits of PKA can then access their targets for phosphorylation. These include HSL, which is phosphorylated at two serine residues in the regulatory region, and perilipin, which is phosphorylated at six serine residues, three in the regulatory region and three in the carboxy terminus. Newer molecules that have been discovered to serve important roles in the lipolytic process include ATGL, which is thought to be a phosphoprotein but whose mode of activation is as yet not understood. Activation of PKA also leads to p38 MAP kinase in rodent and human adipocytes. One of the consequences is the regulation of genes involved in driving the process of mitochondrial thermogenesis. (See color insert.)

ERK pathway appears to account for between 15 and 20% of total lipolysis [166, 168]. Pharmacological analyses suggest that at low catecholamine concentrations essentially all lipolysis is activated by PKA, while the ERK1/2 pathway may be most significant at higher concentrations of epinephrine and norepinephrine. By contrast there is as yet no apparent involvement of the p38 MAPK pathway in β -agonist-stimulated lipolysis. While we have yet to establish what the functional consequences of p38 MAPK activation are in white adipocytes, the transcriptional program of mitochondriogenesis and thermogenesis, including the activation of the UCP1 gene, appears to depend upon the combined stimulation of cAMP/PKA, the p38 α MAPK, and the recruitment and p38-dependent activation of PPAR gamma coactivator-1 α (PGC-1 α) [23, 169]. Based upon what is also known about PGC-1 α to orchestrate the program of mitochondriogenesis [170], it is also very likely that the increased expression and activation of PGC-1 α that occurs as a consequence of β AR stimulation [171, 172] will explain this aspect of SNS-stimulated thermogenesis. Reiterating an earlier concept, there is still much to be learned about the molecular events by which the SNS drives the production and maintenance of thermogenically active brown adipocytes.

27.7 SUMMARY

The relative degree of adipose tissue responsiveness to catecholamines reflects the balance of multiple forces, each of which are shaped by a combination of genetic and environmental factors. On the genetic side are the developmentally programmed patterns and density of innervation into various adipose depots and the amounts and types of the specific adrenergic receptors and their metabolic targets that are expressed on the adipocytes within different depots. Within the latter aspect is whether the receptors are stimulatory (β ARs) or inhibitory (α ARs) for lipolysis, their coupling efficiency to the downstream signaling molecules, and the relative kinetic activity of those regulated enzymes, scaffolding proteins and transcription factors. Finally there is the genetic profile that predisposes preadipocytes toward the progression of the brown versus white adipocyte phenotype. On the environmental side, diet contributes to the relative degree of adipose tissue response to catecholamines by altering the hormonal and metabolic signals that control important adipocyte genes. These include the adrenergic receptors themselves, the kinase pathways they regulate, and hence also the ability to determine decisions about fat storage versus thermogenesis.

With our increased understanding of the SNS circuitry from the CNS to adipose tissue and the newer appreciation of signaling pathways that are activated in adipocytes by catecholamines, we are positioned for the future to better define the molecular and genetic regulatory points that determine adipose tissue sensitivity to dietary and other environmental forces and to establish the underlying basis for the molecular decisions that drive a cell to become a white or brown fat cell. Such new insights that will be gained as a result will hopefully generate new therapeutic targets and approaches against the obesity epidemic in our midst.

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ANTIOBESITY PHARMACOTHERAPY: CURRENT TREATMENT OPTIONS AND FUTURE PERSPECTIVES

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28.1	Introduction	815
28.2	Pharmacotherapy of Obesity	816
28.2.1	Historic Perspectives of Antiobesity Drugs	817
28.2.2	Current Obesity Therapies	818
28.2.2.1	Sibutramine	818
28.2.2.2	Orlistat	820
28.3	Antiobesity Drugs under Development	821
28.3.1	General Strategies for Obesity Drug Development	822
28.3.2	Antiobesity Compounds in Advanced Development	822
28.3.2.1	Axokine	822
28.3.2.2	Rimonabant	824
28.3.3	Antiobesity Compounds with Central Mechanisms in Early Clinical Development	828
28.3.3.1	5-HT _{2C} Receptor Agonists	828
28.3.3.2	MC4 Receptor Agonists	828
28.3.3.3	MCH Receptor-1 Antagonists	829
28.3.3.4	CCK _A Receptor Agonists	830
28.3.3.5	PYY3–36 Peptide Agonists	831
28.4	Future Perspectives	831
	References	833

28.1 INTRODUCTION

Obesity is an ongoing epidemic, and its prevalence rates are steadily rising in many industrialized nations [1]. Obesity and overweight are also becoming a major concern in developing nations due to improved living standards and changes in life styles. In

the United States, the obesity rate has risen from 23% (1988–1994) to 31% (1999–2000) in the last decade, and >60% of adults are considered overweight or obese [2]. Obesity rates are also on the rise in children and adolescents, affecting 16% of 12–19-year-olds in the United States according to recent survey [3]. The obesity rate is higher in females and certain ethnic populations, such as American Indians, Hispanic Americans, and Pacific Islanders [2].

Obesity increases the likelihood of death from all causes by 20% and is associated with a number of chronic conditions, including coronary artery disease, stroke, type 2 diabetes, heart failure, dyslipidemia, hypertension, reproductive and gastrointestinal cancers, gallstones, fatty liver disease, osteoarthritis, and sleep apnea. As a consequence of increased prevalence of childhood obesity, type 2 diabetes is becoming the most common type of diabetes in school children [4]. The economic burden of obesity-related illness is substantial and accounts for nearly 30% of health care spending increase since 1987 in the United States [5].

Obesity is a chronic condition that results from complex interactions of genetic and environmental factors that summate to produce a chronic positive energy balance. Although the specific underlying causes of cumulative weight gain are poorly understood, increased consumption of highly palatable, caloric-dense foods combined with sedentary lifestyles are thought to be the primary factors responsible for the onset of the disease. The central nervous system (CNS) plays an important role in maintaining energy homeostasis by integrating afferent and efferent signals to regulate energy supply and expenditure (Fig. 28.1). The afferent signals are conveyed by a family of incretins and hormones that signal to the brain in response to nutrient intake, whereas efferent signals regulate short-term food intake as well as long-term energy storage in the form of triglycerides in adipose tissues. While our current understanding of the energy homeostasis system is still fragmented, recent progress in characterizing individual drug targets involved in the process has generated valuable information to guide future obesity drug development. Several excellent reviews, including several in this book (see other chapters in this part), have focused the role of the CNS in regulating energy homeostasis [6–8]. This chapter is not intended to give an extensive review of the obesity research field. Instead, emphasis will be given on obesity drugs currently available in the market as well as the latest progress in the clinics on novel antiobesity therapies and their mechanism of action.

28.2 PHARMACOTHERAPY OF OBESITY

Despite the ongoing obesity epidemics, treatment options for obesity are still quite limited. Nonpharmacological methods of obesity therapy, such as exercise and dietary modifications, have demonstrated short-term efficacy, yet most of the weight loss is regained within one year and almost all within five years due to counter regulation from negative energy balance. Surgical procedures such as gastric bypass and banding have proved to be effective but are reserved for morbid obese people with body mass index (BMI) greater than 40 kg/m² because of financial cost, risk of fatality, and long-term complications such as malabsorptions [9]. To date, orlistat and sibutramine represent the only two drugs approved for the long-term treatment of obesity, but neither drug is well accepted by the obese patients due to limited efficacy and issues of side effects.

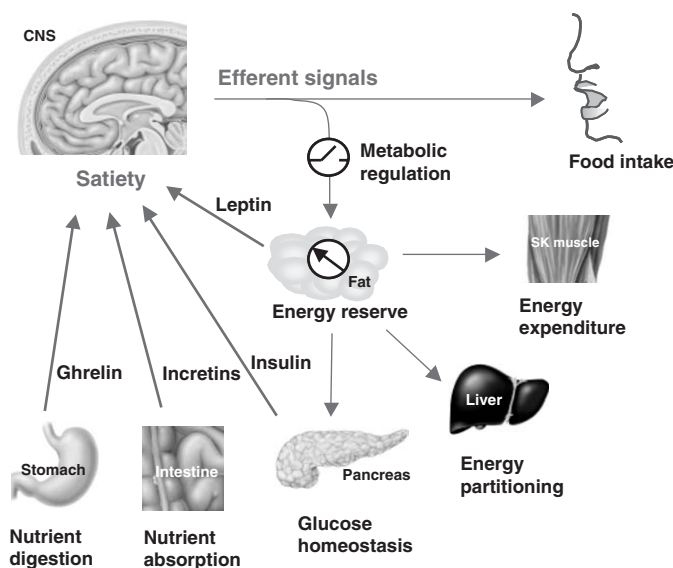


Figure 28.1 Energy homeostasis system. CNS plays an important role in regulating energy homeostasis by integrating signals from the periphery and sending efferent signals that regulate appetite, energy expenditure, and fat storage. Short-term energy balance is regulated in part by ghrelin, insulin, and a family of incretins that sense the level of nutrient intake and send satiety signals to the brain to regulate food intake. Adipose tissue as an endocrine organ secretes a large number of “adipokines,” such as leptin and adiponectin, that gauge the level of long-term energy reserves and signal to the brain and peripheral tissues to regulate both energy expenditure and storage. (See color insert.)

28.2.1 Historic Perspectives of Antiobesity Drugs

The obesity drug development has suffered numerous setbacks and failures in the past due to serious side effects and low efficacy associated with drug treatments, which resulted in a general reticence toward obesity pharmacotherapy. There are many reasons to account for the side effects, but many of the centrally acting anorectic drugs, including sibutramine, are derivatives of β -phenylethylamine. Phenylethylamine is the core structure of several neurotransmitters, including dopamine, norepinephrine (NE), and epinephrine. Consequently, all the catecholaminergic drugs share a side-effect profile that is consistent with their sympathomimetic mechanism of action and have the potential to increase blood pressure and heart rate.

Ephedrine was the first antiobesity drug derived from the Chinese plant *Ephedra sinica*, which led to the chemical synthesis of the amphetamines in the 1930s [10]. However, the abuse tendency toward this class of drugs inspired the synthesis of related drugs that were free of addiction, including phentermine, diethylpropion, and mazindol in the 1960s. Phentermine and diethylpropion are β -phenethylamine derivatives, while mazindol is a tricyclic compound. These drugs demonstrated reasonable clinical efficacy in reducing body weight in short-term studies [11–13]. While this class of drugs showed low abuse potential, they had predominant effects on releasing or blocking the reuptake of noradrenaline in hypothalamic neurons,

which resulted in unpleasant side effects, such as dry mouth, insomnia, and dizziness [14].

Fenfluramine, a racemic mixture, was synthesized in the 1960s by the introduction of a trifluoromethyl group into the phenethylamine ring [15]. The D-isomer of fenfluramine (dexfenfluramine), which has a greater specificity for serotonin (5-HT) release and reuptake inhibition, was found to be more efficacious in reducing food intake [16]. Dexfenfluramine was developed in the 1970s and was licensed in the United States in 1996 with commercial name of Redux for the treatment of obesity. In large-scale clinical trials, the international dexfenfluramine study (INDEX) that involved more than 24 centers, the compound was shown to double the chances of achieving a >10% weight loss when combined with behavioral therapy [17].

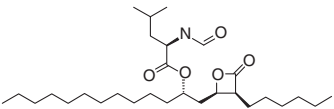
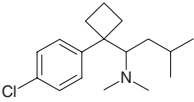
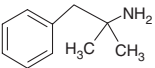
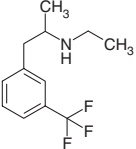
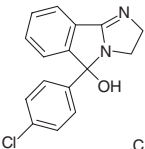
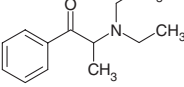
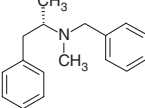
Then came the realization that a combination of phentermine and fenfluramine, also known as Phen-Fen, was far more efficacious due to the different mechanism of action of the two drugs. While amphetamines stimulate the release of dopamine and noradrenaline within the lateral hypothalamus, fenfluramine is believed to inhibit the reuptake of 5-HT in presynaptic neurons terminating within the paraventricular nucleus of the hypothalamus. Redux was one of the most widely prescribed diet drugs in the United States. In 1996, its sales along with fenfluramine were over 20 million prescriptions. The most common side effects include diarrhea and dry mouth. However, it was soon realized that fenfluramine usage was associated with pulmonary hypertension (PPH), a rare but potentially fatal condition [18]. People who used the drug over a longer period are at greater risk. Redux was withdrawn from the market in September 1997 because it was linked with the occurrence of symptomatic valvular heart disease (unrelated to PPH) [19] as well as PPH. Echocardiography demonstrated unusual valvular morphology and regurgitation with features similar to those seen in carcinoid or ergotamine-induced disease.

The major medicines approved and used to treat obesity today and some of the medications approved by the Food and Drug Administration (FDA) and then withdrawn from the marketplace are summarized in Table 28.1. Currently, the only two drugs approved and prescribed for the long-term (more than a year) treatment of obesity are (i) orlistat (Xenical), a gastrointestinal lipase inhibitor that reduces dietary fat absorption, and (ii) sibutramine (Meridia), a centrally acting appetite suppressant that displays its effect through inhibition of both NE and 5-HT reuptake and also by weakly inhibiting dopamine reuptake. In addition, a small set of catecholaminergic appetite suppressants are approved and used for the short-term (up to six-month) treatment of obesity. They all act centrally by modulating the concentrations of monoamine neurotransmitters (5-HT and/or NE) at the level of neurotransmitter release or reuptake or both (Table 28.1).

28.2.2 Current Obesity Therapies

28.2.2.1 Sibutramine. Sibutramine, a racemic mixture of enantiomers of cyclobutanemethanamine, was approved by FDA in 1997 for the treatment of obesity. Its pharmacological effects are mediated predominantly through its active amine metabolites, which are 100-fold more potent than the parent compound [20]. Sibutramine and its active metabolites are inhibitors of the reuptake of 5-HT and noradrenaline [21]. Sibutramine only has very weak dopaminergic activity, thus lacking abuse potential. The compound was initially developed for the treatment of

TABLE 28.1 Current and previously FDA-Approved antiobesity drugs

Drug Name	Duration of Treatment	Mechanism of Action	Trade Name	Drug Enforcement Agency Schedule	Chemical Structure
<i>FDA approved for long-term use (> 1 year)</i>					
Orlistat	Long term	Inhibit gastric & pancreatic lipases	Xenical	None	
Sibutramine	Long term	Inhibit 5-HT & NE reuptake	Meridia	IV	
<i>FDA approved for short-term use (<6 months)</i>					
Phentermine	Short term	Stimulate NE release	Adipex-P, FastinObenix, Zantryl	IV	
<i>Withdrawn or rarely used</i>					
Fenfluramine	Withdrawn	Stimulate 5-HT release	Pondimin	IV	
Mazindol	Few weeks	Adrenoreceptor Agonist	Sanorex, Mazanor	IV	
Diethylpropion	Few weeks	Adrenoreceptor Agonist	Tenuate, Dospan	IV	
Benzphetamine	Few weeks	Stimulate NE release	Didrex	III	

depression but demonstrated poor clinical efficacy in phase II studies [22]. It was later resurrected as a potential treatment for obesity when it was noticed that administration of sibutramine resulted in weight loss in depressed patients.

In a 6-month multicenter study, sibutramine treatment caused dose-dependent weight reduction, ranging from 3.9 to 9.4% (5–30 mg) versus 1.2% placebo ($P < 0.05$) [23]. After adjusting for the placebo effect, once-daily sibutramine reduced body weight by 2.8–5.7 kg [24]. Most of the weight loss was observed during the initial three months of treatment. Sibutramine was also evaluated for its effect in maintaining weight loss after an initial 6 months of treatment with 10 mg/day in conjunction with a low-calorie diet. In a 2-year clinical trial involving 605 patients, individuals who lost at least 5% of body weight were randomized to receive either sibutramine 10–20 mg/day or placebo for 18 months. Although there is a large percentage of dropouts (42% sibutramine and 50% placebo), the results indicate that sibutramine helped maintain weight loss for up to two years, as 43% of the sibutramine and 16% of the placebo group had maintained at least 80% of the weight loss after 2 years [25]. Weight loss resulting from sibutramine treatment also improved metabolic parameters in obese population with type 2 diabetes, dyslipidemia, or hypertension [26]. Additional metabolic benefits associated with Sibutramine usage include lower levels of fasting HbA1c, blood glucose, C peptide, urate, insulin, serum triglycerides, and cholesterol [24].

The mechanisms for long-term weight loss from sibutramine treatment are believed to involve modulation of both satiety and metabolic rate. In rodent studies, sibutramine was shown to reduce food intake by decreasing meal duration rather than frequency, suggesting a predominant effect on satiation [27, 28]. Part of the weight loss from sibutramine treatment is attributable to changes in metabolic rates. Rodents treated with sibutramine demonstrated a 30% increase in oxygen consumption accompanied with a 1°C rise in core temperature, which was mediated via the sympathetic nervous system and brown adipose tissue [29]. An increase in energy expenditure has also been demonstrated in humans [30, 31], though the thermogenic effect remains to be confirmed.

Sibutramine appears to be well tolerated, as judged by the safety profile in placebo-controlled studies involving a large population. However, there are general concerns regarding its sympathetic activity that could exacerbate arterial hypertension and promote cardiovascular disease [32]. The most common adverse events associated with sibutramine treatment include elevated blood pressure, dry mouth, anorexia, insomnia, constipation, and headache [24]. Sibutramine-treated patients had a net increase in blood pressure of 1–3 mm Hg and a pulse rate of 4–5 beats/min compared with placebo [24, 33]. Nevertheless, sibutramine has been cleared from the adverse effect of PPH or heart valve disease associated with dexfenfluramine through extensive postmarketing surveillance programs [24, 34]. As a 5-HT reuptake inhibitor, sibutramine is not recommended to patients who take monoamine oxidase inhibitors such as phenelzine and selegiline. The drug should also be avoided in pregnant or breast-feeding women and in patients with a history of cardiovascular disease, hepatic impairment, or severe renal failure.

28.2.2.2 Orlistat. Orlistat (Xenical), also known as tetrahydrolipstatin, is a potent, specific, irreversible inhibitor of both gastric and pancreatic lipases without major effect on other hydrolases such as phospholipase A2, α -amylase, trypsin,

chymotrypsin, and esterase [35]. It is a chemically synthesized derivative of lipstatin, a natural product from *Streptomyces toxytricini* [36]. The drug reduces fat absorption by ~30% and is excreted almost completely by the fecal route. It acts by forming a covalent bond with the active serine site of gastric and pancreatic lipases in the lumen of the gastrointestinal tract, thus preventing these enzymes from hydrolyzing dietary fat into absorbable free fatty acids and monoacylglycerol [37]. Inactivation of the lipases occurs preferentially at the oil–water interface, and the rate of inhibition in the aqueous phase can be accelerated by the presence of bile salts. In support of the requirement of triglyceride hydrolysis for cholesterol absorption, both cholesterol and low-density lipoprotein were significantly reduced, which appeared to be greater than expected for the degree of weight loss in orlistat-treated patients [38].

Orlistat is the only non-centrally acting drug approved for the treatment of obesity to date. It is effective in treating obesity as demonstrated in large multicenter, randomized clinical trials for up to two years [39]. Patients receiving the drug with hypocaloric diet for the first year lost more weight than those receiving placebo (10.3 vs. 6.1 kg and 8.8 vs. 5.8 kg) [39]. The drug is also effective in weight maintenance and in treating diabetic patients in weight reduction and glycemic control. Diabetic patients receiving orlistat for up to a year experienced significant weight loss compared to those with placebo and a reduction of HbA1c levels [40]. Due to its minimal absorption into the systemic circulation, there is little accumulation of orlistat in patients treated with a dosage of 120 mg three times per day for up to two years [41]. As expected, the major side effects are mainly related to gastrointestinal disorders (8–27% the first year and 6–22% the second year) associated with the undigested triglyceride, such as abdominal pain, urgency to defecate, increased flatus, steatorrhea, and diarrhea. One concern for long-term usage of orlistat is its potential side effect on gall bladder function due to a reduction in cholecystokinin (CCK) secretion caused by decreased levels of free fatty acids in the small intestine. However, such concern has not been confirmed by short-term studies [42]. Although additional natural and synthetic inhibitors were identified, their clinical significance in treating obesity has not been reported [36].

It can be envisaged that a combination of orlistat and sibutramine would be more effective or synergistic in treating obesity due their different mechanisms of action. Surprisingly, no additional weight loss was observed in 34 women who completed one year of sibutramine therapy and were then given combination therapy with sibutramine plus orlistat or placebo [43]. In two short-term (12-week) studies, a total of 86 patients were treated with sibutramine, orlistat, or the combination of both drugs. [44, 45]. The combination therapy seemed to be equally effective in terms of weight reduction compared to orlistat monotherapy [44, 45]. Thus, the benefit of the combination therapy in long-term weight management remains to be elucidated in future study involving larger populations of obese patients.

28.3 ANTI-OBESITY DRUGS UNDER DEVELOPMENT

As discussed in the last section, treatment options with pharmacotherapies are very limited in number, and none of the current obesity drugs is well accepted by the general public because of their marginal efficacy and side effects. This is evident from a meta-analysis of all published antiobesity clinical trials which revealed that the

average placebo-subtracted weight loss induced by any FDA-approved drug never exceeded 4.0 kg [46]. Obviously, obesity represents the largest unmet medical need in the United States and a major challenge to human health in coming years. Furthermore, judging from the limited number of antiobesity compounds currently under clinical evaluation in humans, it is obvious that obesity drug development is facing a great urgency. This provides a great challenge and opportunity for both biotechnology and pharmaceutical companies to develop more effective and safer pharmacotherapies.

28.3.1 General Strategies for Obesity Drug Development

The goal of all antiobesity drugs is to induce and maintain a state of negative energy balance until the desired weight loss is achieved. When daily energy intake matches daily energy expenditure, body weight remains constant. If intake exceeds expenditure, then a state of positive energy balance is achieved and body weight will increase. Conversely, reduced fat mass will occur as a result of negative energy balance.

Based on these principles there are four general strategies to induce a negative energy balance in obese subjects and to develop antiobesity drugs: (i) appetite suppressants to reduce food intake by targeting central components to stimulate anorexigenic signals or to block orexigenic signals; (ii) inhibitors of nutrient digestion and absorption to reduce energy entry into the body through a peripheral gastrointestinal mechanism; (iii) stimulators of fat mobilization and utilization to reduce fat deposition; and (iv) enhancers of energy expenditure to increase thermogenesis by uncoupling of fuel metabolism from the generation of adenosine triphosphate (ATP), thereby dissipating energy as heat.

28.3.2 Antiobesity Compounds in Advanced Development

28.3.2.1 Axokine. Axokine is an analog of ciliary neurotrophic factor (CNTF) currently being evaluated as a potential treatment for obesity. Axokine carries a C-terminal truncation and a substitution of glutamine at position 63 with an arginine of the natural CNTF (Fig. 28.2). As a result of the modification, Axokine is fivefold more potent than the parent CNTF molecule in both *in vitro* neuronal survival assays and *in vivo* studies [47]. CNTF is a 22-kDa neurocytokine expressed by glial cells in peripheral nerves and in the CNS. It was initially discovered more than 20 years ago for its ability to promote survival of ciliary neurons [48]. CNTF is structurally and functionally related to members of the cytokine family, including leukemia inhibitory factor, interleukin 1 and 6, and oncostatin M. The biological function of CNTF is mediated by binding to its receptor CNTFR α , leading to the activation of the JAK/STAT pathways [47]. CNTF is implicated in survival of neuronal cells and in neural responses to injury [49]. The cytokine may also play a role in initiating an acute-phase response in liver cells, maintaining embryonic stem cells in an undifferentiated state, as well as producing a myotrophic effect on denervated skeletal muscles of mice. Increased level of CNTF was observed in the CNS as a response to CNS injury, trauma, sepsis, and cancer, a set of clinical conditions associated with loss of appetite.

The antiobesity potential of CNTF was discovered serendipitously during a clinical trial in which recombinant CNTF was used to treat amyotrophic lateral

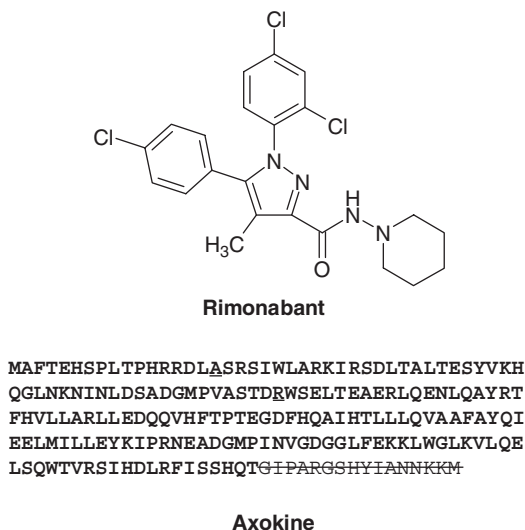


Figure 28.2 Molecular structure of rimonabant and peptide sequence of Axokine. Rimonabant is a selective CB1 receptor antagonist and inverse agonist in late-stage clinical development for the treatment of obesity. Axokine is an CNTF analog that carries a C-terminal truncation (highlighted by a cross-out line) and substitutions of amino acid (highlighted by underline) at position 17 (serine to alanine) and 63 (glutamine to arginine), which resulted with significant improvement of potency of the natural molecule.

sclerosis when anorexia and weight loss were observed in patients treated with the recombinant protein. The anorexigenic effects were later confirmed in experiments using rodent models of obesity. CNTF treatment of genetically obese (*ob/ob*) mice, which lack functional leptin, was found to reduce adiposity, hyperphagia, and hyperinsulinemia associated with the leptin deficiency [50, 51]. In the *db/db* mice, which lack functional leptin receptors, CNTF also reduced obesity-related phenotypes [50].

As a member of cytokine family, CNTF shares overlapping features with leptin but also demonstrates some distinct mechanisms. CNTF and leptin act on the arcuate and paraventricular nuclei of the hypothalamus [52], and the two cytokines share a similar pattern in receptor distribution within hypothalamic nuclei involved in feeding [51]. Both cytokines were shown to downregulate the appetite-stimulating signals, including NPY (neuropeptide Y), AGRP (agouti-related protein), and GABA (γ -aminobutyric acid), while concurrently upregulating the activity of anorexigenic signals POMC (pro-opiomelanocortin) and CART (cocaine and amphetamine-related transcript). Suppression of NPY signaling in the hypothalamus by CNTF did not involve upregulation of leptin [52], suggesting a direct action on hypothalamic NPY neurons or on neural circuits that regulate NPY signaling in the hypothalamus. However, the two cytokines also demonstrate different mechanisms of action. In contrast to leptin that attenuated the fasting-induced increases in corticosterone secretion and AGRP messenger RNA (mRNA) expression, CNTF administration had no effect on corticosterone secretion or AGRP mRNA expression [53]. Such differences offer an explanation why Axokine, but not leptin, remained

effective in *db/db* mice, MC-4 receptor-deficient mice, and mice with diet-induced obesity, which are partially resistant to the actions of leptin [50–52, 54].

In short-term randomized phase II clinical trials involving 170 patients, obese patients who received 0.3–2 µg/kg for 12 weeks lost significantly more weight than the placebo group. Forty-six percent of drug-treated patients lost at least 10 lb compared to 5% of the placebo group. Axokine is also effective in treating obese type 2 diabetics. Diabetic patients ($n = 157$) who received 0.5 or 1 µg/kg of Axokine and dietary counseling for 12 weeks lost an average of 6.5 lb, while those who received counseling and placebo lost 2.5 lb [55, 56]. In a pivotal phase III study enrolling more than 2000 patients at 65 sites in the United States, patients were randomized to receive 1 µg/kg Axokine or placebo for the first 12 months, followed by a 12-month open-label extension phase, during which all study subjects received Axokine. The results showed that a greater proportion of Axokine-treated patients lost at least 5% of their initial body weight compared with placebo-treated patients (25.1% vs. 17.6%, $p < 0.001$). Participants receiving Axokine experienced a greater average weight loss than those receiving placebo (6.2 lb vs. 2.6 lb, $p < 0.001$), and 11.3% of the treated group lost 10% body weight compared to 4.2% in the placebo group. However, Axokine-associated weight loss was limited by the development of antibodies, which occurred in two-thirds of the treated group after 3 months of therapy. On an adjusted basis, Axokine-treated participants who completed 1 year without developing antibodies achieved greater average weight loss (12.6 lb vs. 4.5 lb, $p < 0.001$), higher proportion of at least 5% (46% vs. 24%, $p < 0.001$), or 10% (24% vs. 6.6%, $p < 0.001$) weight reduction of initial body weight [57].

Axokine treatment was generally well tolerated. Adverse events were generally characterized as mild to moderate and no pattern of serious or severe adverse events emerged. The most notable adverse effects as compared with placebo were injection site reactions, nausea, and cough, which were largely characterized as mild. The initial concerns that CNTF may have deleterious effects common to cachectic cytokines, such as fever, hepatic acute-phase protein responses, and muscle wasting, are not supported by the clinical data. In animal studies, CNTF administration did not induce toxicity malaise, illness, or taste aversion. These findings suggest that CNTF is functionally more related to leptin than a typical cytokine. However, precautions must be drawn on the propensity of CNTF in inducing weight loss without posttreatment overeating and immediate rebound weight gain. Further investigation is warranted to investigate the underlying mechanisms and to rule out potential pathophysiological consequences associated with this unique feature of the drug. Finally, whether Axokine will ever be used as a drug to treat obesity rests on the future efforts in improving the potency of CNTF without causing antibody responses.

28.3.2.2 Rimonabant. Rimonabant (Acomplia) is a selective antagonist of endocannabinoid receptor type 1 (CB1) currently under development by Sanofi-Aventis (formerly Sanofi-Synthelabo) for the indication of obesity [58]. Cumulative information indicates that activation of CB1 by endogenous cannabinoids, such as anandamide, increases appetite. The drug, which has progressed to phase III development, works by blocking endogenous cannabinoid binding to neuronal CB1 receptors, thus offering a unique therapeutic approach to appetite control and weight gain. The drug

also has potential as a treatment for smoking cessation because the endocannabinoid system is also involved in the body's response to tobacco dependence.

The ability of *Cannabis sativa* (marijuana) to stimulate hunger has been realized for centuries, although the underlying mechanisms have not been uncovered until recently by the cloning and characterization of the endogenous receptors, generation of knockout mice deficient in the receptors, and chemical synthesis of agonists and antagonists of the receptors. The cannabis activity is mediated by two cannabinoid receptors (CB1 and CB2) that are members of G-protein-coupled receptor (GPCR) superfamily. The CB1 receptor was cloned from the screening of an "orphan" GPCR with several possible ligands [59]. The receptor is widely distributed in the CNS and is present at highest density in basal ganglia, cerebellum, hippocampus, and cortex [60]. The CB1 receptor is also present in the peripheral nervous system and several peripheral organs, whereas CB2 receptors are mostly restricted to the immune tissues and cells [61]. Both receptors have been shown to couple to the $G_{i/o}$ proteins through which they modulate the activity of adenylyl cyclase, mitogen-activated protein kinases, and in the case of CB1 receptors the voltage-activated Ca^{2+} channels and inwardly rectifying K^+ channels, upon binding of a cannabinoid agonist [62]. The receptors are also able to crosstalk with other intracellular signal transduction pathways mediated by neurotransmitter receptor systems [63].

The presence of specific receptors mediating the actions of cannabis and its derivatives stimulated research interests in the identification and characterization of endogenous ligands for CB receptors. The first endogenous cannabinoid, arachidonyl ethanolamide, was identified from the porcine brain in 1992 and was named anandamide, from the Sanskrit word "ananda" for internal bliss [64]. Despite lacking structural similarity with Δ^9 -tetrahydrocannabinol (THC), the main psychoactive ingredient of marijuana identified in the 1960s, anandamide recognizes the same CB receptors and is able to reproduce most of the biological activities of marijuana in cell-based assays and in rodents [65]. Additional endogenous CB agonists have been identified, all of which are derivatives of polyunsaturated fatty acids, collectively termed endocannabinoids [66, 67]. Among these endocannabinoids, 2-arachidonoyl-glycerol (2-AG), initially identified in canine gut, is the most abundant endocannabinoid in the brain [68]. Endocannabinoids, like neurotransmitters, are released from neurons following membrane depolarization and Ca^{2+} influx into the cells and are inactivated by reuptake and hydrolysis [62].

The therapeutic potentials of the cannabinoid system have been exploited by the development of synthetic agonists and antagonists. Information accumulated from the last decade of studies on plant cannabinoids and the endocannabinoid system paved a way to develop more innovative therapeutics. In addition to stimulation of appetite, CB1-mediated behavioral effects include ataxia, analgesia, hypothermia, euphoria, short-term memory deficits, and cognitive impairments [65, 69], whereas CB2 receptors modulate immune responses and attenuate nociceptive behavior in models of acute and persistent pain [69]. Moreover, cannabinoids seem to exert neuroprotective function by protecting neurons from hypoxic and traumatic injury. The dynamic functional roles of the endocannabinoid system open opportunities to develop selective agonists and antagonists to treat a spectrum of diseases. Several natural and synthetic compounds are currently under development for the treatment of severe head injury, analgesia, inflammation, multiple sclerosis, pain, glaucoma, and cancer [69, 70].

The effect of cannabinoids on food intake is clearly related to their distribution and direct action in the hypothalamus. Both CB1 and endocannabinoids are present at high levels in the hypothalamus, the brain region most directly involved in the regulation of appetite and food intake. Activation of CB1 receptor modulates the release of neurotransmitters, including GABA, dopamine, noradrenaline, glutamate, and 5-HT, all of which play a role in regulating feeding and satiety. Diet restriction in mice causes a significant reduction in levels of dopamine and 5-HT, which can be partly restored by low-dose anandamide administration [71, 72]. Furthermore, endocannabinoid levels are elevated in the hypothalamus and limbic forebrain of food-deprived mice and in *ob/ob* mice and *fa/fa* rats (mutant rats lacking leptin) with defective leptin signaling, whereas leptin treatment of normal mice and *ob/ob* mice reduces anandamide and 2-AG in the hypothalamus [73, 74].

The effect of cannabinoids on appetite has been demonstrated in both rodents and human studies. Administration of THC or anandamide caused hyperphagia in animals fed palatable food [75]. In support of a role of cannabinoids in orosensory reward of food, increased motivation for sucrose intake and beer consumption was observed in rats treated with a cannabinoid agonist [76, 77]. Similarly, a significant reduction in food intake was observed in rodents treated with the CB1 receptor antagonist SR 141716A (rimonabant) [73]. Furthermore, mice with targeted deletion of CB1 receptor are lean and resistant to diet-induced obesity [78].

Humans have a long history of use and abuse of cannabis. Marijuana was used to treat appetite loss in India in ancient times. As the most widely used illegal drug in Western society, marijuana smoking is associated with increased appetite. Such effect was confirmed in a series of human studies under standardized conditions. When given orally, THC treatment stimulated food intake in both fasted and fed conditions, although the effect was more profound in fed subjects [79]. Marijuana smoking caused significantly increased daily food intake and consumption of snack foods [80, 81]. Both heavy and casual marijuana users had a significant increase in caloric intake and weight gain during a five-day period of marijuana smoking [82]. The use of cannabinoid to treat anorexia was exploited with the approval of dronabinol (Marinol), a synthetic THC, by the FDA in 1985 to treat chemotherapy-induced nausea and vomiting. The same drug was approved by the FDA in 1992 to treat human immunodeficiency virus (HIV)-induced wasting syndrome. Acquired immunodeficiency syndrome (AIDS) patients treated with dronabinol experienced an increase in appetite which resulted in stabilization of weight or modest weight gain. The positive effect of dronabinol on weight gain was also observed in patients suffering from Alzheimer's disease.

The potential of cannabinoids in controlling appetite has been exploited in recent years to treat obesity by the development of CB1 receptor antagonists. Rimonabant, a selective CB1 receptor antagonist, is one of several compounds currently under development for the treatment of obesity. Rimonabant is believed to possess both antagonist and inverse agonist activity [83]. In comparison to pure antagonists that block the receptor activation by an agonist, an inverse agonist has the ability to decrease the constitutive level of receptor activation in the absence of agonist. Rimonabant may be more efficacious in inhibiting CB1 receptor activity than a pure antagonist, since CB1 and CB2 receptors have been shown to possess constitutive signaling activity in the absence of ligand [84]. In preclinical studies, rimonabant was shown to cause a decrease in appetite and body weight in genetically obese rodents

and mice with diet-induced obesity [73]. The underlying mechanism is believed to be parallel to or downstream from that of leptin pathways, since the compound is able to cause weight loss in both *ob/ob* and *db/db* mice as well as in mice with targeted deletion of NPY [73] but not in CB1 knockout mice [78]. The antiobesity effect of rimonabant was associated with an improvement in serum lipid profile, activation of thermogenesis, and enhanced leptin and insulin sensitivity [85, 86].

The promising preclinical findings with rimonabant have been confirmed in studies in humans. Results from a 16-week phase II trial showed that the drug was well tolerated at all the doses tested, and the treatment produced significant weight loss in obese patients. Phase III trials of rimonabant in obesity (RIO) involving over 6000 obese patients in North America and Europe were recently carried out to compare rimonabant at doses of 5 and 20 mg with placebo with respect to weight reduction and prevention of weight gain for a period of 2 years [87]. Phase III results from RIO-North America, an international multicenter randomized trial involving 3040 overweight/obese patients, demonstrated that treatment with rimonabant (20 mg) significantly lowered weight, reduced abdominal fat, diminished cardiovascular risk factors, and decreased metabolic disorders in this patient population. The benefits achieved in the first year with rimonabant 20 mg were sustained in the second year. Patients treated with rimonabant 20 mg for the full 2 years experienced a reduction in waist circumference by 8 cm (3.1 in.) versus 4.9 cm (1.9 in.) for rimonabant 5 mg and 3.8 cm (1.5 in.) for the placebo group ($p < 0.001$). A significantly higher proportion of patients who received treatment with rimonabant 20 mg lost more than 5% (62.5% vs. 33.2%, $p < 0.001$) and 10% (32.8% vs. 16.4%, $p < 0.001$) of their initial body weight than those on placebo. Similar results were achieved with phase III RIO-Europe involving 1507 overweight and obese patients.

Metabolic parameters were also significantly improved in patients treated with rimonabant 20 mg, as indicated by a significant improvement in high-density lipoprotein (HDL) cholesterol and triglyceride levels when compared to those treated with placebo. A 27% increase in HDL cholesterol was achieved in patients on rimonabant 20 mg/day for one year, compared to 17.3% in the placebo group ($p < 0.01$). Triglycerides were reduced by 10.6% in patients on rimonabant 20 mg, compared to a 6.6% increase in the placebo controls. Although diabetic patients were not included in the study, patients on rimonabant 20 mg had significantly improved their insulin sensitivity, as indicated by significant lower serum insulin levels in patients treated with 20 mg rimonabant when compared to those on placebo ($p < 0.05$) during a 2-h glucose tolerance test. Additional trials are in progress to evaluate the efficacy and safety of rimonabant in obese patients with concomitant type 2 diabetes and dyslipidemia.

Results from the clinical data reported so far indicate that rimonabant was well tolerated. Overall discontinuation rates for adverse events in the first year of the study were 7.2, 9.4, and 12.8% in the placebo, rimonabant 5-mg, and rimonabant 20-mg groups, respectively. Although mice with targeted deletion of CB1 receptor displayed reduced locomotive activity, increased ring catalepsy, and hypoalgesia [88], these effects were not reported from patients treated with rimonabant. Additionally, no major side effects have been reported with regards to anxiety/depression symptoms between the treated and control groups. The most common side effects include nausea, diarrhea, and dizziness, which appeared to be mild and transient. However, the long-term adverse effects of rimonabant treatment remain to be

investigated, since the cannabinoid system plays dynamic functional roles in memory, learning, cognition, reproduction, and neuroprotection [70, 89]. Specifically, the effect of the drug on quality of sleep should be monitored closely, since rats treated with rimonabant displayed an increase in wakefulness at the expense of slow-wave and REM sleep [90].

28.3.3 Antiobesity Compounds with Central Mechanisms in Early Clinical Development

Currently, a number of antiobesity compounds have advanced to early clinical trials (summarized in Table 28.2). These compounds target different pathways of the energy metabolism, including CNS regulation of appetite.

28.3.3.1 5-HT_{2C} Receptor Agonists. The 5-HT system has been implicated in weight regulation, which is mediated primarily by the activation of 5-HT receptor 2C (5-HT_{2C}) [91]. 5-HT_{2C} receptor gene expression is found in the hypothalamus, a brain area critical to weight regulation. Mice with targeted deletion of the 5-HT_{2C} receptor develop chronic hyperphagia and obesity [92], whereas both mice and rats treated with 5-HT_{2C} receptor agonists demonstrated hypophagia and weight loss. Several of the clinically effective anorectic agents including sibutramine and fenfluramine exert their hypophagic and weight loss effects via 5-HT_{2C} receptor activation [21].

Weight gain associated with antipsychotic drugs such as clozapine appears linked to a high-affinity binding to 5-HT_{2C} receptor and these drugs function as antagonists [93]. Furthermore, genetic polymorphism of the 5-HT_{2C} gene promoter is associated with susceptibility to antipsychotic-induced weight gain [94]. Finally, treatment of obese patients with *m*-chlorophenylpiperazine (mCPP), a selective 5-HT_{2C} agonist, suppressed appetite and caused weight reduction [95]. These observations have led researchers to conclude that selective 5-HT_{2C} receptor agonists may have the potential to be effective antiobesity agents. More importantly, it is hoped that selective 5-HT_{2C} agonists may retain the clinical efficacy of fenfluramine without the adverse effects, since it is generally believed that the adverse effects of fenfluramine are caused by its activation of 5-HT_{2B} and other related receptor subtypes. Currently, several 5-HT_{2C} selective agonists have advanced to early clinical trials [96]. However, caution must be taken in evaluating the safety of this class of compounds because of their possible functional relationship with fenfluramine.

28.3.3.2 MC4 Receptor Agonists. The melanocortin-4 receptor (MC4R), a centrally expressed GPCR, is essential for the maintenance of long-term energy balance in humans [97]. Its main ligand is α -melanocyte-stimulating hormone (α -MSH), one of several peptide cleavage products of the POMC molecule [98]. MC4R is found in the hypothalamic nuclei implicated in energy homeostasis. The expression levels of POMC mRNA are regulated by energy status, that is, reduced during fasting and restored by refeeding [99]. Mutations of the MC4R and POMC genes are the most common genetic causes of human obesity that is often accompanied by red hair, early onset obesity, and congenital hypocortisolism [100, 101]. MC4R polymorphism is associated with late-onset obesity in humans [102]. Similarly, mice with targeted deletion of MC4R demonstrate hyperphagia and overt obesity [103]. Furthermore,

administration of MC4R agonists suppresses appetite in wild-type mice, but not in MC4R knockout mice [104].

Although major efforts have been made in recent years in the development of small molecular agonists for MC4R, it proved difficult to mimic the action of MSH with small molecules in terms of efficacy and specificity [105, 106]. As an alternative, potent α - and β -MSH peptide analog with improved half-lives have also been developed [107, 108]. Peripheral administration of MSH analogs results in reduction in food intake and body weight in rodent studies [107, 109]. Currently, several α -MSH peptide analog have advanced to early clinical trials, as shown in Table 28.2. These compounds hold promises for the next generation of antiobesity treatment, since the melanocortin system represents the most validated drug target for obesity.

28.3.3.3 MCH Receptor-1 Antagonists. Melanin-concentrating hormone (MCH) is a cyclic peptide originally isolated from the teleost fish as a pituitary hormone that

TABLE 28.2 Antiobesity drugs in different stages of human clinical trials.

Drug Name	Development Phase	Mechanism of Action	Molecular Nature	Marketing Company
Axokine	III	Nerve growth factor (CNTF), centrally acting	Peptide	Regeneron
Rimonabant	III	CB-1 antagonist, centrally acting	Sm molecule	Sanofi-Synthelabo
Ecopipam	III	Dopamine D ₁ /D ₅ receptor antagonist, centrally acting	Sm molecule	Schering-Plough
ISIS-113715	II	PTP1B antisense Oligonucleotide	ISIS	
AC137	II	Amylin analog	Peptide	Amylin
ATL-962	II	Lipase inhibitor	Sm molecule	Alizyme
AOD9604	II	hGH 177-191	Peptide	Metabolic
5-HT _{2C}	II	5HT _{2C} agonist, centrally acting	Sm molecule	Arena, Roche/Vernalis Bristol-Myers Squibb
MCH	I	MCH-R antagonist, centrally acting	Sm molecule	Neurogen, Tularik
MC4 agonist	I	Agonist, centrally acting	Sm molecule	Millennium, Neurocrine
PYY3-36	I	Agonist, centrally acting	Peptide	Nastech
DGAT	I	Enzyme inhibitor	Sm molecule	Bayer

regulates skin color. The biological function of MCH in humans is mediated by two GPCRs known as MCHR1 and MCHR2. The two receptors share similar tissue distribution patterns within the CNS [110], although MCHR1 is more abundant and exhibits a wider distribution pattern than MCHR2, whose function has yet to be determined due to its absence in rodents [111]. Cumulative evidence suggests that MCH plays an important role in maintaining energy homeostasis by regulating satiety, energy expenditure, and thermogenesis [112]. The expression of both MCH and MCHR1 is upregulated by genetic obesity and food deprivation [113, 114]. Intracerebroventricular (ICV) injection of MCH stimulates food intake in rats, which can be functionally antagonized by α -MSH, an inhibitor of appetite [115, 116]. Chronic infusion of MCH or a peptide agonist induces hyperphagia and body weight gain [117], whereas targeted deletion of MCH suppresses appetite and adiposity [118]. Additionally, mice deficient in MCHR1 expression are hyperphagic and resistant to diet-induced obesity [119, 120].

Further evidence regarding a role of MCH in regulating energy homeostasis comes from development and testing of nonpeptide antagonists in animal models of obesity. Administration of MCH antagonist compounds orally or by injection decreases food intake and body weight and blocks the onset of diet-induced obesity [121–123]. Several compounds are currently being tested in humans in early clinical trials (Table 28.2). While MCH antagonism holds promise as a viable treatment for obesity, major concerns arise from potential side effects associated with MCH inactivation, since MCH is believed to regulate various brain activities, such as behavior, olfaction, memory, and emotions. Mice with targeted deletion of MCHR1 or rodents treated with MCHR1 antagonists produced antidepressive and anxiolytic effects in animal models [121, 124]. Consistent with wide tissue distribution of MCHR1 receptors [110], mice with targeted deletion of MCHR1 also exhibited osteoporosis [125], increased heart rate [126], and cognition deficits [127]. However, it is not clear whether these abnormal phenotypes will be recapitulated by an MCHR1 antagonist compound. Furthermore, precautions have to be taken when interpreting data from rodents in relation to human physiology of MCH-mediated events due to the absence of MCHR2 in rodents.

28.3.3.4 CCK_A Receptor Agonists. CCK is predominantly expressed in the duodenum and is secreted primarily in two forms, CCK-33 and CCK-8, in response to nutrient intake [128]. Its secretion is regulated by dietary fat content, suggesting a sensory role of the hormone for dietary fat [129]. CCK exerts its biological effects by binding and activating two GPCRs known as CCK_A (CCK-1) and CCK_B (CCK-2). CCK_A is primarily localized in the gastrointestinal system, whereas CCK_B is found within the CNS [130]. CCK has a variety of gastrointestinal functions, including pancreatic secretion, gallbladder contraction, intestinal motility, and gastric emptying [131]. CCK expression is also found in the brain, where it functions as a neurotransmitter regulating reward behavior, memory, and anxiety as well as satiety [132, 133].

A role of CCK in regulating satiety was proposed more than three decades ago when peripheral administration of CCK was found to decrease food intake [134, 135]. The anorexigenic effect is primarily mediated by the CCK_A receptor [136, 137]. Administration of CCK_A selective agonists causes a dose-dependent suppression of food intake, whereas CCK_A antagonists stimulated food intake in rodents [135, 138,

139]. In contrast, CCK_B agonists and antagonists have no effect on feeding [140]. Consistent with these findings, CCK deficiency in the Otsuka Long-Evans Tokushima fatty (OLETF) rat contributes to the onset of the obese phenotype [141]. Like all the natural incretins, the native CCK peptide has a very short half-life. Major efforts have been made in development of selective peptide analog for the two receptors, since CCK_A and CCK_B have very different functional roles [130]. The therapeutic potentials of CCK in obesity have been explored in recent years through the development of peptide agonists [142, 143]. Among them, GI-181771, a peptide agonist selective for CCK_A receptors developed by GSK, was shown to cause significant weight loss in humans after eight weeks of treatment. The development of this compound has recently been discontinued after disappointing data from phase III clinical trials [144]. In addition, the major effects of CCK on gastric emptying and on other neurological behaviors are also major concerns for this class of drugs.

28.3.3.5 PYY3–36 Peptide Agonists. PYY is a potent central orexigenic peptide initially isolated from colonic extracts [145]. It shares high sequence homology with NPY and pancreatic polypeptide (PP) [146]. PYY is secreted from the intestinal L cells of the gastrointestinal tract in response to nutrient intake [147] and signals the brain to regulate satiety by acting through NPY receptor-2 (Y2R) in the hypothalamic arcuate nucleus [148]. The peptide is present in two circulating forms, PYY1–36 and PYY3–36 [149]. PYY3–36 is produced by the cleavage of the N-terminal Tyr-Pro residues by dipeptidyl peptidase IV and can cross the blood–brain barrier freely by nonsaturable mechanisms [150].

The regulatory role of PYY has been validated in both rodent models of obesity and obese human patients [151]. Peripheral administration of PYY3–36 has been shown to acutely inhibit food intake in rodents [152], but not in Y2R knockout mice [148]. The orexigenic effect of PYY3–36 remains intact in mice deficient in either POMC or MC4R [153, 154], indicating that the melanocortin system is not obligatory for the actions of PYY3–36. Circulating PYY levels are suppressed in morbid obese humans but are reversed following gastric bypass surgery, suggesting PYY deficiency may contribute to the pathogenesis of obesity [155–157]. The anorectic effects of PYY3–36 have also been confirmed in human studies. When given intravenously to both normal-weight and obese volunteers, PYY3–36 has been shown to reduce food intake by more than 30% [156]. Moreover, cumulative caloric intake remained significantly reduced 24 h postinfusion. Unlike problems with leptin, the anorectic effect was preserved in obese subjects despite much lower levels of PYY in this group of patients [156], suggesting replacement of circulating PYY levels might be an effective antiobesity therapy. Efforts have been made to develop PYY analog with improved potency that can be delivered by nasal spray as a treatment for obesity. However, the pharmaceutical potential of PYY analog remains to be corroborated by data from human clinical trials since conflicting data have been presented on its orexigenic effects in rodents [158].

28.4 FUTURE PERSPECTIVES

The discovery of leptin signaled a milestone in obesity research that stimulated a more general interest in obesity drug development. The last decade has been

described as the golden years of obesity research, which resulted in identification and characterization of numerous obesity drug targets. However, this has not translated into a rich pipeline of antiobesity drugs, as judged by the number of compounds in late-stage clinical trials and the high attrition rate of clinical compounds. The latest candidates that have failed in phase III trials for obesity include a CCK agonist (GlaxoSmithKline), topiramate (Johnson and Johnson), which acts through biogenic amines, and ecopipam (Schering Plough), a dopamine D₁/D₅ receptor antagonist, due to poor efficacy or therapeutic profiles. Treatment options for obesity lag behind the treatment options for other chronic diseases but are comparable to those in the cardiovascular area four decades ago. Hypertension at that time was treated with diet, surgery, and drugs with substantial side effects, as obesity is treated today. This analogy suggests that the development of innovative medicines for the treatment of obesity is only at its beginning.

To be ultimately successful, the development of an antiobesity drug has to overcome major hurdles, such as issues of limited efficacy, toxicity, and strong resistance of the system to perturbation. Like many chronic conditions, the underlying causes for obesity are quite heterogeneous as a result of genetic and environmental interactions. The network that regulates energy homeostasis is extremely complex and involves the integration of both central and peripheral mechanisms (Fig. 28.1). Although obesity is defined as an excessive accumulation of triglycerides in the adipose tissue, there are probably as many obesity subtypes as in the case of cancers. The dream of developing a single treatment for all obese patients is not realistic, as reflected by the fact that only a limited population of patients is responsive to any given treatment in most clinical trials when corrected for the placebo effect [46]. Although our current understanding of the factors that differentiate these subtypes is still very limited, future development of biomarkers would offer a potential solution to the problem.

The obesity drug market has encountered frequent problems in the past with toxicity issues. Since the launch of the first antiobesity drug dinitrophenol (a synthetic thermogenic drug) in the 1930s, almost every drug treatment has encountered adverse effects that have either resulted in termination or limited usage of those drugs. To be effective, obesity drugs must interfere with the biochemistry of energy metabolism that is essential for survival. Furthermore, in addition to regulating energy metabolism, many of the obesity drug targets also serve other physiological functions, which can lead to serious side effects when disrupted. Thus, all current and future antiobesity drugs must be evaluated by their relative benefit to risk ratios, for which the general public needs to be educated. Drawing a parallel with recent progress in monoclonal antibody therapy for the treatment of cancers, the side effects associated with obesity drugs will be improved with future progress in our understanding of the underlying causes.

Mammals have evolved multiple and redundant pathways that safeguard energy metabolism for survival under both feast and famine conditions. When one pathway is altered with an antiobesity drug or diet, powerful compensation and feedback from other pathways will occur, which limits long-term efficacy in weight loss. Furthermore, obesity is like any other chronic disease, such as diabetes, in which hyperglycemia would occur upon discontinuation of treatment. Thus, all the antiobesity drugs currently available are effective mainly in the initial period of treatment, and continuous treatment is needed for long-term weight maintenance.

From this perspective, the lack of rebound from the treatment of CNTF is quite unique, and its underlying mechanisms need to be uncovered to benefit future drug development.

The decoding of the human genome sequence combined with recent development of new technologies, such as bioinformatics, interference RNA (RNAi), and QMR, has provided unprecedented opportunity for rational design of novel obesity therapeutics. At the same time, it is realized that human obesity is rarely caused by the failure of a single gene as displayed in many rodent models but is a malady resulting from the interaction of a genetic proclivity and environment. This raises a considerable challenge to the development of future drugs that act on a single molecular target. Although interest continues in a single agent that could combine the clinical effects of the phentermine/fenfluramine combination without the side effects, the future marketplace belongs to the combination therapy of antiobesity drugs that combine central and peripheral effects to achieve maximum benefit. This requires the next generation of antiobesity drugs to target different aspects of energy metabolism, from central regulation of appetite to peripheral effects on nutrient absorption and energy storage and expenditure [159]. It remains a hope that the serendipitous nature of antiobesity drug discovery will be replaced in the near future with rational design benefiting from the explosion of information in obesity research accumulated in the last decade. The recent successful development of rimonabant has set an example for the new paradigm and may signify the dawning of a new era in obesity therapeutics.

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INDEX

- AB1-42:
 - Alzheimer's disease, invertebrate models, 579–582
- ABT-418 nicotinic agonist:
 - dementia disorders and, 467–468
- ACEA 1021/1031 agents:
 - stroke management, 358
- Acetylcholinesterase inhibitors:
 - dementia disorders and, 463–465
- Acquired immunodeficiency syndrome (AIDS):
 - alcohol abuse and, 716
 - cholinergic system, 711–712
 - clinical features, 694–695
 - dementia and, 711–712
 - drug abuse and, 712–716
 - methamphetamine/cocaine, 713–715
 - opioid drugs, 715–716
 - excitatory amino acid neurotransmitters, 711
 - future neuropharmacology issues, 718
 - lipid metabolism alterations, 707
 - neurodegenerative diseases and, 716–718
 - neuropathology, 695–704
 - cell death cascades, 704–707
 - apoptosis, 704–705
 - excitotoxicity, 705–706
 - neural progenitor cells, 706–707
 - oxidative stress, 706
 - chemokines in, 697–698
 - neurodegenerative mechanisms, 699
 - neuropharmacology
 - features of, 694–695
 - neurotoxic proteins, 699–704
 - glycoproteins gp120 and gp41, 699, 701
 - Nef protein, 703
 - neurobiology of, 700
 - Rev protein, 704
 - Tat protein, 701–703
 - Vpr protein, 703
 - Vpu protein, 704
 - nigrostriatal system, 707–711
 - dopamine mediators, 709–711
 - pathogenesis, 695
- Acute motor axonal neuropathy (AMAN):
 - pathology, 614
- Acylated peptide hormones:
 - ghrelin receptor ligands, 766–768
- Adaptor proteins:
 - tyrosine kinase receptor signaling, 243–245
- Addictive disorders:
 - hypocretin/orexin system, 135
- Adenosine A_{2A} receptors:
 - neuroinflammation
 - purinergic receptor modulation and, 643–644
 - Parkinson's disease treatment, 505–506
- Adenosine deaminase (ADA) deficiency:
 - neuroinflammation and, 643–644
- Adenosine triphosphate (ATP):

- adipose tissue cell types and depots, 786–789
- ischemic brain injury, 351–353
- Adenylyl cyclase:
 - adipose tissue signaling and, 802–803
- Adhesion molecules:
 - inflammation mechanisms, 624–625
- Adipocytes:
 - ghrelin modulation, 775
 - leptin secretion and, 735–736
 - proliferation, sympathetic nervous system innervation and, 797–799
 - white vs. brown molecular features, 787–789
- Adipose tissue:
 - catecholamine signaling mechanisms, 801–803
 - cell types and depots, 786–789
 - future research issues, 804
 - glyceroneogenesis, 799–801
 - sympathetic nervous system innervation, 789–797
 - adipocyte proliferative capacity, 797–799
 - anterograde tract-testing, SNS to WAT, 792–797
 - retrograde tracing neuroanatomical studies, 790–792
 - white vs. brown adipocytes, 787–789
- Adrenergic agonists:
 - attention-deficit hyperactivity disorder therapy, 299–300
 - autism spectrum disorders, 330
 - Tourette's syndrome therapy, 276–277
- Adrenergic neurotransmitters:
 - cataplexy therapy
 - animal models, 109–110
- Adrenocorticotropin hormone (ACTH):
 - for secondary generalized epilepsy, 424
- Adrenoreceptors:
 - white adipose tissue innervation, 789–797
- Adult respiratory distress syndrome (ARDS):
 - adenosine pathway and purinergic receptor modulation, 644
- Advanced sleep phase syndrome (ASPS):
 - circadian rhythms, melatonin receptor modulation, 58
- AF102B agonist:
 - dementia disorders and, 466
- Affective disorders:
 - autism spectrum disorder and, 333
 - circadian rhythms and, 15–17
- Afferent neurones:
 - hypocretins at, 130
- Aftereffects:
 - circadian rhythms, 6
- Age of onset modifiers:
 - idiopathic Parkinson's disease, 542
- Aggression:
 - autism spectrum disorders, 334
- Agomelatine:
 - melatonin receptor targeting, 57–58
 - depression therapy, 59–61
- Agouti-related peptide (AgRP):
 - antiobesity therapy
 - axokine, 823–824
 - hypocretin/orexin system and, 132–134
- Alcohol abuse:
 - HIV neuropharmacology and, 716
 - idiopathic Parkinson's disease and, 544
- ALE-0540:
 - nerve growth factor inhibitor, 232
- Alemtuzumab:
 - multiple sclerosis therapy, 677–679, 686
- Alzheimer's disease:
 - diagnostic criteria, 462
 - invertebrate models, 579–582
 - neuropathies and, 227–228
- Alzheimer's disease (AD):
 - acetylcholinesterase inhibitors and, 463–465
 - glutamate receptor ligands, 469–471
 - muscarinic receptor drugs and, 465–469
 - nicotinic agonists, 469
 - therapeutic targets, 471–473
- Amantidine:
 - autism spectrum disorders, 332
 - Parkinson's disease treatment, 496–497
- α -Amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA):
 - antiepileptic drugs and
 - topiramate, 416–417
 - dementia disorders and, 470
 - neurogenesis and, 208
 - neurotrophic factor synthesis and, 232
 - stroke and
 - ischemic brain injury, 351–353
 - receptor antagonists, 361–363
- Amisulpiride:
 - Tourette's syndrome therapy, 276
- AMP-activated kinase (AMPK):
 - ghrelin effects and, 773

- Amphetamines:
 attention-deficit hyperactivity disorder therapy, 296–298
 narcolepsy therapy
 adverse effects, 98
 cerebrospinal fluid hypocretin-1 assessment, 84
 drug interactions, 98, 100
 excessive daytime sleepiness and, 90–100
 histocompatibility human leukocyte antigen testing, 84
 molecular targeting, 93–94
 polysomnography, 83
 stroke therapy and, 377
 traumatic brain injury and, 453–454
- β -Amyloid peptide:
 in HIV patients, 717–178
 neurodegenerative disease
 immunization modulation of, 644–645
- Amyloid precursor protein (APP):
 Alzheimer's disease, invertebrate models, 579–582
 in HIV patients, 717–178
- Amyotrophic lateral sclerosis (ALS):
 neurotrophic factor therapy and, 225–227
- Anesthesia:
 hypocretin/orexin system, 136
- Angiogenesis:
 prokineticins and, 169–170
- Animal models:
 antiepileptic drugs
 topiramate, 416–417
 circadian rhythms
 mammalian models, 10–13
 nonmammalian models, 6–9
 human autoimmune neuropathies, 615
 invertebrate models
 neurodegenerative disease:
 Alzheimer's disease, 579–582
 Caenorhabditis elegans system, 568–569
 Drosophila melanogaster, 569
 early research, 567–568
 future applications, 582–583
 genetic and molecular pathways, 568
 Huntington's disease trinucleotide repeats, 577–579
 Parkinson's disease
 dopamine neuron cell death, 569–577
 myelin disorders
 experimental allergic encephalomyelitis, 611–612
 narcolepsy
 hypocretin-1 deficiency, 86
 modafinil therapy, 102–103
 stroke and
 global and focal ischemia, 353–354
 NMDA receptor antagonists, 355–356
- Anterograde tract-tracing:
 sympathetic nervous system innervation
 white adipose tissue, 792–797
- Antiadhesion molecules:
 stroke management, 369–370
- Antiapoptotic signaling:
 p75 neurotrophin receptor, 246–247
- Anticholinergic agents:
 Parkinson's disease treatment, 496, 502
- Anticonvulsant drugs. *See also* Antiepileptic drugs (AEDS)
- Antidepressants:
 cataplexy therapy, 114–115
 historical overview, 106–107
 second- and third-generation, 108
 tricyclic antidepressants, 107–109
 circadian rhythms and, 18–20
 melatonin receptors, therapeutic targeting
 of, 58–61
 neurogenesis and, 209–210
 sleep effects of, 17–18
- Antiepileptic drugs (AEDs). *See also* Anticonvulsant drugs
 autism spectrum disorder therapy, 332–333
 epilepsy, 404–405
 calcium channels, 413–414
 clinical efficacy, 417–418
 clinical prediction markers, 420
 combination therapy, 423–424
 development and testing, 418–420
 felbamate interactions, 414–415
 GABA_A receptor and, 411–412
 generalized absence epilepsy, 421
 localization-related epilepsy, 421–423
 mechanisms of action, 408–410
 metabotropic glutamate receptors, 413
 mixed actions, 414–418, 422
 secondary generalized epilepsies, 424
 selection criteria, 421–424
 sodium channel modulation, 411
 special population requirements, 424–425

- synaptic vesicle protein, 414
- topiramate interactions, 415–418
- toxicity detection, limitations of, 420–421
- molecular targeting, 409–410
- pharmacokinetic characteristics, drug interactions and serum levels, 422
- Antigen-presenting cells (APC):
 - neuroinflammation, 626
 - astrocytes and, 627
- Antigen-specific T cells:
 - inflammation mechanisms, 625
- Antiglutamate agents:
 - Parkinson's disease treatment, 496–497
- Antihistamines:
 - sedative/hypnotics and, 188
- Anti-inflammatory agents:
 - stroke management, 367–371
 - traumatic brain injury and, 447–449
- Anti-NoGo agents:
 - stroke therapy, 377–378
- Antibesity agents:
 - current development of, 821–831
 - axokine, 822–824
 - CCK_A receptor agonists, 830–831
 - future research issues, 831–833
 - MC4 receptor agonists, 828–829
 - MCH receptor-1 antagonists, 829–830
 - orlistat, 820–821
 - PYY3-36 peptide agonists, 831
 - rimonabant, 824–828
 - serotonin receptor 5-HT_{2C} agonists, 828
 - FDA-approved drugs, 819
 - future research issues, 832–833
 - historic perspective on, 817–818
 - orlistat, 820–821
 - sibutramine, 818–820
- Antioxidants:
 - neuroinflammation and, 636–637
 - Parkinson's disease therapy, 504–505
 - stroke management, 371–372
- Antiproliferative/proliferative mechanisms:
 - ghrelin modulation, 776
- Antipsychotic agents:
 - attention-deficit hyperactivity disorder therapy, 301
 - autism spectrum disorders
 - atypical agents, 326–330
 - typical agents, 325–326
- Anxiety and anxiety disorders:
 - Parkinson's disease treatment and, 500–501
- Aplysia californica*:
 - circadian rhythmicity, 9
- Apomorphine:
 - Parkinson's disease treatment, 493–494, 505
- Apoptosis mechanisms:
 - HIV neuropathology, 704–705
 - neuroinflammation and modulation of, 637–638
 - stroke management, 372–373
- Appetite regulation:
 - ghrelin effects, 772–773
 - hypocretin/orexin system, 132–134
 - leptin signaling and, 740–743
 - prokineticins and, 168–169
- Arachidonic acid pathways:
 - neuroinflammation and, 630
 - pharmacological activation of, 634–635
- Aripiprazole:
 - autism spectrum disorders, 327–328
- AR juvenile parkinsonism:
 - parkin* gene mutations and, 530–532
- Aromatic amino acid decarboxylase (AADC):
 - Parkinson's disease neurochemistry, 481–483
- Arousal:
 - hypocretin/orexin system, 139–144
 - cholinergic systems, 143–144
 - dopaminergic systems, 143
 - feeding and motivation integration, 144
 - histaminergic systems, 142–143
 - lateral hypothalamic neurons, 140–141
 - noradrenergic systems, 141–142
 - serotonergic systems, 142
 - sleep and circadian rhythms:
 - chronopharmacology, 15–23
 - functional importance, 4
 - future research issues, 23–24
 - mammalian structural models, 10–13
 - nonmammalian structural models, 6–10
 - properties, 4–6
 - superchiasmatic nucleus period and phase, 13–15
- Artemin (ARTN):
 - discovery of, 221–223
- Aschoff's rules:
 - circadian rhythms, 5–6

- Astrocytes:
 - HIV neuropathogenesis and, 697
 - neuroinflammation and, 627
 - cytokine/chemokine expression, 632
- Ataxin-3:
 - Huntingdon's disease, invertebrate model, 578–579
- Atomoxetine:
 - attention-deficit hyperactivity disorder therapy, 299
- Atorvastatin:
 - multiple sclerosis therapy, 667
- Attention-deficit hyperactivity disorder (ADHD):
 - clinical management issues, 301–302
 - diagnostic criteria, 291–292
 - evaluation, 292–293
 - pharmacotherapy, 293–301
 - adverse effects, 298
 - amphetamines, 296–298
 - antipsychotics, 301
 - atomoxetine, 299
 - bupropion, 300–301
 - clonidine/guanfacine, 299–300
 - dexmethylphenidate, 296
 - methylphenidate hydrochloride, 294–296
 - modafinil, 301
 - nonstimulants, 299–301
 - stimulants, 293–298
 - tricyclic antidepressants, 299
 - venlafaxine, 301
 - Tourette's syndrome comorbidity, 266–268, 276–277
 - treatment rational, 292
- Autism spectrum disorders:
 - aggression/self-injury, 334
 - anxiety and depression, 334
 - interfering repetitive behaviors, 334
 - motor hyperactivity and inattention, 333–334
 - overview, 320–321
 - pharmacology, 321–333
 - adrenergic agonists, 330
 - amantidine, 332
 - antiepileptic drugs, 332–333
 - antipsychotics, 325–328
 - atypical antipsychotics, 326
 - clomipramine, 322
 - clonidine, 330
 - clozapine, 326
 - d-cycloserine, 332
 - dopaminergic medications, 325–330
 - fluoxetine, 323–324
 - fluvoxamine, 322–323
 - glutamatergic medications, 331–332
 - guanfacine, 330–331
 - haloperidol, 325
 - lamotrigine, 332
 - methylphenidate, 329–330
 - mirtazapine, 331
 - olanzapine, quetiapine, ziprasidone, and aripipazole, 327–328
 - pimozide, 325–326
 - risperidone, 326–327
 - serotonergic medications, 321–325
 - sertraline, citalopram, escitalopram, and paroxetine, 324–325
 - stimulants, 328–330
 - venlafaxine, 331
 - syndromes within, 333–335
- Autoimmune disorders:
 - demyelinating disorders of PNS, 613–615
 - human autoimmune neuropathies, 613–614
 - Tourette's syndrome and, 268–269
- Autonomic effects:
 - hypocretin/orexin system, 134–135
- Autosomal-dominant (AD) Parkinsonism:
 - genetic mutations in, 527
- Autosomal-recessive (AR) Parkinsonism:
 - genetic mutations in, 527
- Avonex:
 - multiple sclerosis therapy, 684–685
- Axokine:
 - antiobesity therapy, 822–824
- B7 molecule:
 - inflammation and, 631
- Baclofen:
 - Tourette's syndrome, 277
- Barbiturates:
 - as epilepsy therapy, 423
 - GABA_A receptor binding site, 181
 - structure and function, 184–187
- Basal ganglia:
 - Parkinson's disease
 - neurochemistry, 482–483
 - Tourette's syndrome, 269–270
- Bcl-2 family proteins:
 - stroke management, 373
- Behavior inhibition/activity:
 - autism spectrum disorders, 334
 - hypocretin/orexin system, 144

- melatonin modulation of circadian rhythms, 54–57
- Tourette's syndrome therapy, 277–278
- Benserazide:
 - Parkinson's disease treatment, 484–487
- Benzedrine:
 - attention-deficit hyperactivity disorder therapy, 296–298
- Benzhexol:
 - Parkinson's disease treatment, 496
- Benzodiazepines:
 - circadian rhythms and, 20–21
 - epilepsy efficacy, 417–418
 - as epilepsy therapy, 423
 - GABA_A receptor binding site, 180–181
 - sedative/hypnotic functions, 187–188
 - stroke management, 362
- Benztropine:
 - Parkinson's disease treatment, 496
- Betaseron:
 - multiple sclerosis therapy, 674–675
- Biological antagonists:
 - neurotrophic factors as, 231
- Blood-brain barrier (BBB):
 - HIV neuropathogenesis and, 696–697
 - inflammation and, 624–625
 - pharmacological activation, 633
- Blood-derived inflammatory cells:
 - neuroinflammation, 628
- BMAL1 protein:
 - circadian rhythms
 - animal models, 12
 - melatonin receptors and, 50–51
- Body composition:
 - ghrelin modulation, 775
- Bone morphogenetic proteins (BMP):
 - stroke and, 375–377
- Bone resorption and formation:
 - ghrelin modulation, 776
- Brain:
 - immunoreceptors, inflammation and, 631–632
 - sedative/hypnotic action sites in, 181–184
- Brain-derived neurotrophic factor (BDNF):
 - amyotrophic lateral sclerosis and, 226–227
 - cognitive function and, 228
 - depression and, 229–230
 - discovery of, 221–223
 - HIV neuropathology
 - Tax protein and, 705
 - indirect modulators of, 232
 - neurogenesis and, 206, 224
 - neuroinflammatory mechanisms, 641
 - neuronal maturation regulation, 224
 - neurotrophins and, 238
 - obesity and weight control, 229–230
 - p75 neurotrophin receptor signaling, 246–247
 - pain perception and management, 229
 - Parkinson's disease and, 228–229
 - structure of, 240
 - synaptic function and, 239
- Brain injury:
 - neurotrophic factors and, 229–230
 - stroke and
 - classification, 350
 - mechanisms of, 351–353
 - poststroke repair approaches, 375–379
 - repair approaches, 377–379
 - traumatic
 - characteristics and classification, 443–446
 - future research issues, 454
 - neural regeneration, 452–453
 - neuroplasticity, 453–454
 - neuroprotective agents, 446–451
 - anti-inflammatory agents, 447–449
 - free-radical scavengers, 447
 - neuroactive steroids/neurosteroids, 450–451
 - neurotransmitter agonists/antagonists, 449–450
- Breast cancer resistance protein (BCRP):
 - drug-resistant epilepsy, 425–426
- Bromocriptine:
 - Parkinson's disease treatment, 488, 490
- Brown adipose tissue (BAT):
 - classification, 786–789
 - glyceroneogenesis control, lipolysis and thermogenesis, 799–801
 - leptin and energy expenditure, 744
 - molecular features, 787–789
- Bupropion:
 - attention-deficit hyperactivity disorder therapy, 300–301
 - narcolepsy therapy, 103–104
- CAAT-enhancing binding proteins (C/EBP):
 - leptin secretion and expression of, 735–736
- Cabergoline:
 - Parkinson's disease treatment, 491
- Caenorhabditis elegans*:
 - neurodegenerative disease model

- Alzheimer's disease, 580–582
 - genetic and molecular pathways, 568–569
- Huntingdon's disease trinucleotide repeats, 577–579
- Parkinson's disease cell death, 570–577
- Caffeine:
 - idiopathic Parkinson's disease and, 544
 - narcolepsy therapy, 104–105
 - Parkinson's disease and, 505
- Calcium channels:
 - hypocretin/orexin system, 132
 - neuroinflammation and, 642
 - stroke and
 - ischemic brain injury, 351–353
 - neuronal channel blockers, 366–367
 - subunits
 - antiepileptic drug modulation, 413–414
 - suprachiasmatic nucleus, 13–15
 - T-type
 - antiepileptic drug modulation, 413
- cAMP response element binding (CREB)
 - protein:
 - cognitive function and, 228
 - HIV neuropathology and apoptosis and, 705
 - Huntingdon's disease, invertebrate model, 579
 - leptin secretion and expression of, 735–736
 - melatonin receptors
 - supersensitization, 54
- Cannabinoid receptors:
 - antiobesity therapy, 825–828
 - neuroinflammation and
 - modulation mechanisms, 641–642
- Carbamazepine:
 - development and testing, 419
 - epilepsy efficacy, 417–418
 - focal/localization-related epilepsy therapy, 421, 423
- Carbidopa:
 - Parkinson's disease treatment, 484–487
- Carbonic anhydrase:
 - topiramate and, 416–417
- Cardiovascular system:
 - ghrelin effects, 774–775
- Caspase inhibitors:
 - HIV neurotoxicity
 - Vpr protein, 703
 - stroke management, 372–373
- Cataplexy:
 - defined, 82
 - mazindol therapy, 103
 - narcolepsy pathophysiology, 85
 - human leukocyte antigen and, 85–86
 - pharmacological treatment
 - antidepressants, 114–115
 - future antiepileptics, 113–114
 - historical overview, 106–107
 - monoamine oxidase inhibitors, 111
 - neurotransmission, animal studies, 109–110
 - receptor subtypes, 110–111
 - second- and third-generation antidepressants, 108
 - sodium oxybate, 112
 - tricyclic antidepressants, 107–109
- Catecholamine signaling:
 - in adipose tissues, 801–803
- Catechol-*O*-methyl transferase (COMT):
 - Parkinson's disease
 - neurochemistry, 481–483
- CCK_A receptor agonist:
 - antiobesity therapy, 830–831
- CD9 protein:
 - structure and function, 608–609
- CD40 molecule:
 - inflammation and, 631
- CD80 molecule:
 - inflammation and, 631
- CD86 molecule:
 - inflammation and, 631
- Cell autonomy:
 - Circadian rhythmicity, 8
- Cell death cascades:
 - human immunodeficiency virus
 - neuropathology, 704–707
 - apoptosis, 704–705
 - excitotoxicity, 705–706
 - neural progenitor cells, 706–707
 - oxidative stress, 706
- Cellular migration:
 - neural inflammation and
 - pharmacological activation, 633
- Central nervous system (CNS):
 - HIV neuropathogenesis and, 696–697
 - inflammation mechanisms in, 622–625
 - melatonin receptors
 - circadian rhythm modulation, 54–57
 - clock genes and, 50–51
 - desensitization, 52–53

- historical perspective, 38–40
- melatonin production, 40–41
- molecular pharmacology, 42–44
- molecular structure, 41–42
- overview, 37–38
- regulatory mechanisms, 51–54
- signaling mechanisms, 44–46
- supersensitization, 54
- suprachiasmatic nucleus, 46–54
 - circadian inputs and outputs, 46–47
 - receptor localization, signaling, and function, 47–50
- as therapeutic targets, 57–61
 - circadian rhythms, 58
 - depression, 58–61
 - sleep, 57–58
- CEP-1347 compound:
 - Parkinson's disease and, 229
- Ceramide production:
 - HIV lipid metabolism and, 707
- Cerebroside/sulfatide lipids:
 - in myelin, 594–596
- Cerebrospinal fluid (CSF):
 - hypocretin/orexin system
 - narcolepsy and, 138–139
 - narcolepsy evaluation
 - hypocretin-1 assessment, 84
- Cerostat/Aptiganel:
 - stroke management, 359
- Cerovive:
 - stroke management, 371–372
- Charcot-Marie-Tooth:
 - neurotrophic factor therapy, 227
- Chemokines:
 - HIV neuropathogenesis and, 697–699
 - inhibitors
 - stroke management and, 369
 - neuroinflammation and, 629–632
 - receptor-directed pharmacological approaches to, 633–634
- Chemotherapy:
 - multiple sclerosis
 - mitoxantrone, 676–677
- Cholecystokinin (CCK):
 - antiobesity therapy, 830–831
 - leptin signaling and feeding regulation, 743
- Cholesterol:
 - in myelin, 594–596
- Choline acetyltransferase:
 - HIV-associated dementia and, 711–712
- Cholinergic systems:
 - hypocretin/orexin system
 - arousal mechanisms, 143–144
 - neurogenesis and, 208–209
- Chromosome mapping:
 - hypocretin/orexin system, 127
- Chronic inflammatory demyelinating polyneuropathy (CIDP):
 - myelin pathology, 613–614
- Chronopharmacology:
 - circadian rhythms, 15
- Ciliary neurotrophic factor (CNTF):
 - amyotrophic lateral sclerosis, 226–227
 - antiobesity therapy
 - axokine, 822–824
 - depression and, 229–230
 - therapeutic applications, 226
- Circadian rhythms:
 - antidepressant effects on, 18–20
 - chronopharmacology, 15–23
 - functional importance, 4
 - future research issues, 23–24
 - mammalian structural models, 10–13
 - melatonin receptor modulation, 54–57
 - therapeutic targeting, 58
 - neurotrophins and, 239
 - nonmammalian structural models, 6–10
 - prokineticin regulation, 167–168
 - properties, 4–6
 - superchiasmatic nucleus
 - inputs and outputs, 46–47
 - period and phase, 13–15
- Cisplatin-induced neuropathy:
 - neurotrophic factors and, 227
- Citalopram:
 - autism spectrum disorders, 324–325
- Claudin protein family:
 - structure and function, 608
- "Clinically isolated syndrome" (CIS) of demyelination:
 - betaseron therapy and, 675
- Clinical markers:
 - antiepileptic drug efficacy, 420
- Clock genes:
 - melatonin receptors and, 50–51
 - supersensitization function, 54
 - prokineticin regulation, 167–168
- Clock neurons:
 - synchronization and modulation, 8–9

- CLOCK protein:
 circadian rhythms
 animal models, 12
 melatonin receptors and, 50–51
- Clomipramine:
 attention-deficit hyperactivity disorder therapy, 299
 autism spectrum disorders, 322
- Clonazepam:
 Parkinson-related sleep disturbance, 504
- Clonidine:
 attention-deficit hyperactivity disorder therapy, 299–300
 autism spectrum disorders, 330
 circadian rhythms and, 19–20
 Tourette's syndrome therapy, 274–276
- Clozapine:
 autism spectrum disorders, 326
- CNS 1102 antagonist:
 stroke and, 357
- Cocaine:
 HIV/AIDS and, 713–715
- Cocaine- and amphetamine-regulated transcript (CART) peptides:
 antiobesity therapy
 axokine, 823–824
 leptin signaling and feeding regulation, 741–743
- Cockroach:
 circadian rhythms in, 10
- Coenzyme Q₁₀:
 Parkinson's disease treatment, 504–505
- Cognitive function. *See also* Dementia in HIV patients, 717–718
 neural system diversity and, 461–463
 neuropathies and, 227–228
 traumatic brain injury and, 446
- Comorbid conditions:
 Tourette's syndrome, 266–268
- Connexins:
 structure and function, 609
- Conotoxins:
 stroke management
 calcium channel blocker, 366–367
- Constipation:
 Parkinson's disease and, 503
- Cortical neurons:
 HIV neuropathology
 apoptosis and, 705
 Tourette's syndrome, 270
- Corticosteroids:
 multiple sclerosis acute relapse, treatment of, 672–673
 neurogenesis and, 206–207
 traumatic brain injury and, 448–449
- Corticotropin-releasing factor (CRF):
 hypocretin/orexin system, 135
- Cortisol:
 circadian rhythms and release of, 19–20
- Costimulatory molecules:
 inflammation and, 631
- Cryptochrome genes:
 melatonin receptors, 50–51
- Cyclic adenosine monophosphate (cAMP):
 melatonin receptors
 supersensitization, 54
 neuroinflammation and
 modulating agents, 635–636
- Cyclic guanosine monophosphate (cGMP):
 neuroinflammation and
 modulating agents, 635–636
- 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP):
 structure and function, 603–604
- Cyclooxygenase (COX1) inhibitors:
 neuroinflammation
 arachidonic acid/prostaglandin pathways, 634–635
- Cyclooxygenase (COX2) inhibitors:
 neuroinflammation
 arachidonic acid/prostaglandin pathways, 634–635
 traumatic brain injury and, 448–449
- d*-Cycloserine:
 autism spectrum disorders, 332
- Cysteines:
 melatonin receptor molecular structure, 42
- Cytochrome P450:
 Tourette's syndrome therapy, 274–276
- Cytokines:
 circadian rhythms and, 21–23
 multiple sclerosis therapy
 daclizumab agents, 685–686
 neuroinflammation
 arachidonic acid pathways, 630
 astrocytes and, 627
 minocycline inhibition and, 645–646
 proinflammatory pathway modulation, 638–639
 receptor-directed pharmacological approaches to, 633–634
 structure and function, 628–629

- stroke and
 - ischemic brain injury, 352–353
 - stroke management and inhibition of, 368–369
 - traumatic brain injury and, 447–449
- D₂/D₃ receptors:
 - cataplexy therapy, 110–111
 - novel anticataplectics, 113–114
 - Tourette's syndrome therapy, 276
- Daclizumab:
 - multiple sclerosis therapy, 685–686
- Dardarin mutation:
 - familial Parkinson's disease and mutation of LRRK2 gene and, 538–539
- Daytime sleep studies:
 - narcolepsy evaluation, 83
- D-CPP-ene NMDA antagonist:
 - stroke management and, 356–357, 360
- Death domain:
 - p75 neurotrophin receptor structure and, 241
 - stroke management, 373
- Decahydroisoquinolines:
 - stroke management, 362
- Decarboxylase inhibitor:
 - Parkinson's disease treatment
 - levodopa with, 484–487
- Deep sleep phase syndrome (DSPS):
 - circadian rhythms, melatonin receptor modulation, 58
- Dementia:
 - acetylcholinesterase inhibitors and, 463–465
 - glutamate receptor ligands, 469–471
 - HIV-associated (HAD)
 - classification, 694–695
 - muscarinic receptor drugs and, 465–469
 - nicotinic agonists, 469
 - Parkinson's disease and, 502
 - therapeutic targets, 471–473
- L-Deprenyl:
 - narcolepsy therapy, 104
- Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP), 504–505
- Depression:
 - autism spectrum disorders, 334
 - melatonin receptors, therapeutic targeting of, 58–61
 - neurotrophic factors and, 229–230
 - Parkinson's disease treatment and, 500–501
- Desensitization:
 - melatonin receptors, 52–53
- Desipramine:
 - attention-deficit hyperactivity disorder therapy, 299
- Dexmethylphenidate:
 - attention-deficit hyperactivity disorder therapy, 296
- Diet:
 - epilepsy management and, 427
- Diethylpropion:
 - as anti-obesity agent, 817–818
- Dihydroergocryptine:
 - Parkinson's disease treatment, 491
- 3,4-Dihydroxyphenylacetic acid (DOPAC):
 - human immunodeficiency virus
 - methamphetamine/cocaine abuse and, 714–715
 - nigrostriatal system and, 709
 - Parkinson's disease
 - neurochemistry, 481–483
- Diphasic dyskinesia:
 - Parkinson's disease treatment and, 499
- Disturbed nocturnal sleep:
 - treatment of, 112
- DJ-1* gene:
 - familial Parkinson's disease and, 535–536
- Donepezil:
 - dementia disorders and, 464–465
- Dopamine agonists:
 - human immunodeficiency virus and
 - nigrostriatal system, 708–710
 - Parkinson's disease treatment, 488–494
 - antioxidant effects, 504–505
 - apomorphine, 493–494
 - bromocriptine, 488, 490
 - cabergoline, 491
 - monoamine oxidase type B inhibitor, 494
 - pergolide, 490–491
 - piribedil, 493
 - pramipexole, 491–492
 - ropinirole, 492
 - pharmacology of, 489
- Dopamine neurotransmitters:
 - hypocretin/orexin system
 - arousal mechanisms, 143
 - narcolepsy therapy
 - amphetamine targeting, 92–94
 - adverse effects, 98

- anatomical substrates, 97–98
 - drug interactions, 98, 100
 - EEG arousal and, 94–97
 - modafinil, 102–103
- Parkinson's disease neurochemistry and, 481–483
 - invertebrate models of cell death, 569–577
- Tourette's syndrome, 270–273
- Dopaminergic receptors:
 - autism spectrum disorders
 - medications, 325–330
 - human immunodeficiency virus
 - nigrostriatal system and, 710–711
 - opioid abuse and, 715–716
 - Tourette's syndrome, 266–267
- Dopamine transporters:
 - attention-deficit hyperactivity disorder therapy, 297–298
- Dopaminomimetic agents:
 - Parkinson's disease, 484–496
 - apomorphine, 493–494
 - bromocriptine, 488, 490
 - cabergoline, 491
 - COMT inhibitors, 495
 - dihydroergocryptine, 491
 - dopamine agonists, 488–494
 - entacapone, 496
 - levodopa plus peripheral decarboxylase inhibitor, 484–487
 - levodopa slow-release formulations, 487–488
 - lisuride, 491
 - monoamine oxidase type B inhibitor, 494
 - pergolide, 490–491
 - piribedil, 493
 - pramipexole, 491–492
 - rasagiline, 495
 - ropinirole, 492
 - selegiline, 494–495
 - stalevo, 496
 - tolcapone, 495
- Dose failure:
 - Parkinson's disease treatment and, 499
- Downstream techniques:
 - stroke management, 366–373
- Drosophila* models:
 - Alzheimer's disease, 580–582
 - drug effects in, 7–8
 - Huntingdon's disease trinucleotide repeats, 578–579
 - neurodegenerative disease
 - genetic and molecular pathways, 569
 - Parkinson's disease cell death, 570–577
 - rhythmicity in, 7
- Drug abuse:
 - Drosophila* models, 7–8
 - in HIV/AIDS, 712–716
 - methamphetamine/cocaine, 713–715
 - opioid drugs, 715–716
- Drug discovery:
 - neurotrophic factors and, 230–232
- Drug interactions:
 - narcolepsy therapy
 - amphetamines, 98, 100
- Drug-resistant epilepsy, 425–426
- Dyskinesia:
 - Parkinson's disease treatment and classification of, 499
 - levodopa agents, 487
- Electrical stimulation:
 - epilepsy management, 427
- Electrocoagulation model:
 - stroke-related global and focal ischemia, 353–354
- Electroencephalogram (EEG):
 - epilepsy investigation, 405–407
 - narcolepsy therapy
 - dopaminergic neurotransmission and, 94–95
- Eliprodil:
 - stroke management, 357, 359
- Endocrine system:
 - hypocretin/orexin system, 134–135
- Endothelial cells:
 - neuroinflammation, 627
 - pharmacological activation, 633
- Energy expenditure:
 - leptin and, 743–744
- Entrainment properties:
 - circadian rhythms, 5
- Entrainment stimuli:
 - clock neuron synchronization and modulation, 8–9
- Environmental factors:
 - idiopathic Parkinson's disease and, 543–545
 - Tourette's syndrome, 267–269
- Enzymes:
 - in myelin, 609–610
- Ephedrine:
 - as anti-obesity agent, 817–818

Epilepsy:

- acute seizures and status epilepticus, 427–428
- alternative therapies, 426–427
- antiepileptic drugs, 404–405
 - calcium channels, 413–414
 - clinical efficacy, 418
 - clinical prediction markers, 420
 - combination therapy, 423–424
 - development and testing, 418–420
 - felbamate interactions, 414–415
 - GABA_A receptor and, 411–412
 - generalized absence epilepsy, 421
 - localization-related epilepsy, 421–423
 - mechanisms of action, 408–410
 - metabotropic glutamate receptors, 413
 - mixed actions, 414–418, 422
 - secondary generalized epilepsies, 424
 - selection criteria, 421–424
 - sodium channel modulation, 411
 - special population requirements, 424–425
 - synaptic vesicle protein, 414
 - topiramate interactions, 415–418
 - toxicity detection, limitations of, 420–421
- child-onset absence
 - classification, 405–406
- classification, 404–405
- clinical investigation, 405–408
- diet and, 427
- drug-resistant, 425–426
- electrical stimulation therapy, 427
- focal/localization-related epilepsy
 - classification, 405–406
 - prognosis, 408
 - therapy for, 421, 423
- future research issues, 428–429
- generalized absence form
 - therapy options, 421
- prognosis of, 407–408
- secondary generalized epilepsy syndrome
 - classification, 405–406
 - therapy options, 424
- surgical management of, 426
- temporal lobe epilepsy
 - classification, 405–406
 - surgical treatment, 426

Epinephrine:

- white adipose tissue innervation, 789–797

EPO receptor (EPOR):

- stroke therapy and, 377

ERK1/ERK2 protein kinases:

- tyrosine kinase receptor signal transduction, 243

Erythropoietin (EPO):

- neuroinflammatory mechanisms, 641
- stroke therapy and, 377

Escitalopram:

- autism spectrum disorders, 324–325

Estrogen response element (ERE):

- leptin receptor expression and, 738–739

Estrogens:

- neuroinflammation and, 645
- traumatic brain injury and, 450–451

Ethosuximide:

- development and testing, 419
- epilepsy efficacy, 417–418
- for generalized absence epilepsy, 421
- T-type calcium channel modulation, 413

Excessive daytime sleepiness (EDS):

- idiopathic hypersomnia and, 84
- mazindol therapy, 103
- narcolepsy, 80–82
 - amphetamines, 90–100
 - drug interactions with, 98, 100
 - molecular targeting of, 93–94
 - side effects, 98
 - bupropion, 103–105
 - caffeine, 104–105
 - dopamine neurotransmitters:
 - substrates, 97–98
 - dopamine neurotransmitters and EEG arousal, 94–97
 - future stimulant development, 105–106
 - mazindol, 103
 - modafinil, 100–103
 - nonamphetamine stimulants, 99–105
 - pharmacological treatment, 90–100
 - selegiline, 104

Excitatory amino acid transporters

(EAATs):

- stroke management, 365–366

Excitatory mechanisms in stroke:

- AMPA receptor antagonists, 361–363
- amphetamine and neurotransmitter modulators, 377
- antiadhesion molecules, 369–370
- anti-nogo (IN-1) inhibitors, 377–378
- antioxidants, 371–372
- apoptosis and caspase inhibitors, 372–373
- basic characteristics and symptoms, 348–349

- brain injury with
 - classification, 350
 - mechanisms of, 351–353
 - repair approaches, 377–379
- calcium channel blockers, 366–367
- chemokine inhibition, 369
- cytokine inhibition, 368–369
- decahydroisoguinolines, 362
- down-stream approaches, 367–368
- erythropoietin, 377
- global/focal ischemia
 - animal models, 353–354
- glutamate/glutamatergic receptors,
 - 354–355, 361–366
- glutamate release inhibitors, 366–367
- glutamate transporters, 365–366
- growth factors, 375–377
- GYKI 52466/related benzodiazepines, 362
- inflammatory pathways, 368
- kainate receptor antagonists, 363
- metabotropic glutamate receptors,
 - 363–365
- NBQX/related quinoxalinediones,
 - 361–362
- neuroprotective techniques, 366–373
 - development criteria, 374
- nitric oxide synthase inhibition, 370–371
- NMDA receptor antagonists, 354–359
 - clinical trial data, 358–359
 - competitive/noncompetitive agonists,
 - 356–357
 - glycine site antagonists, 357–358
 - MK-801 compound, 355–356
 - polyamine site antagonists, 357
 - side effects, 359–360
- p38 inhibition, 369
- prevalence and incidence, 349–350
- sodium channel blockers, 367
- sonic hedgehog approach, 378
- stem cell approach, 378–379
- time window issues, 360–361
- upstream techniques, 366–373
- Excitatory neurotransmitters:
 - hypocretin/orexin system, 131–132
- Excitotoxicity cell death:
 - human immunodeficiency virus and,
 - 705–706
- Experimental allergic encephalomyelitis (EAE):
 - natalizumab, 684–685
 - novel therapy for, 678–679
 - pathogenesis, cytokines and, 628–629
 - pathophysiology, 611–612
 - statin therapy, 668
- Extended-release stimulants:
 - attention-deficit hyperactivity disorder
 - therapy, 295
- Extrapyramidal symptoms:
 - Tourette's syndrome therapy, 274–276
- Extrasynaptic receptors:
 - GABA_A receptor structure
 - sedative/hypnotic mechanisms, 180
- Fas-associated death domain (FADD)
 - protein:
 - stroke management, 373
- Fatty acid binding proteins (FABP):
 - protein 2, 603
- Fatty acid metabolisms:
 - overview of, 785–786
- Feeding mechanisms:
 - ghrelin effects, 772–773
 - hypocretin/orexin system, 132–134
 - arousal integration, 144
 - leptin signaling and, 740–743
 - prokineticin regulation, 168–169
- Felbamate:
 - epilepsy efficacy, 417–418
 - ionotropic glutamate receptor
 - modulation, 413
 - for secondary generalized epilepsy,
 - 424
 - target interactions of, 414–415
- Fibroblast growth factors (FGFs):
 - stroke therapy and, 375–377
- FKBP12 protein:
 - multiple sclerosis therapy, 667
- Flenfluramine:
 - as anti-obesity agent, 818
- Fluid extravasation:
 - inflammation and
 - pharmacological activation, 633
- Fluoxetine:
 - autism spectrum disorders, 323–324
 - circadian rhythms and
 - antidepressant effects, 19–20
 - neurogenesis and, 209–210
- Fluvoxamine:
 - autism spectrum disorders, 322–323
 - circadian rhythms and release of, 20
- 4P-PDOT antagonist:
 - melatonin receptor molecular
 - pharmacology, 43–44
 - circadian rhythm modulation, 55–57

- desensitization function, 53
 - in suprachiasmatic nucleus, 49–50
- Free-radical scavengers:
 - traumatic brain injury and, 447
- FTY720 agent:
 - multiple sclerosis therapy, 689
- Functional recovery:
 - stroke therapy and, 375–379
- GABAergic neurons:
 - Parkinson's disease treatment and, 505–506
 - sedative/hypnotic action sites, 184
 - Tourette's syndrome and, 270–273
- Gabapentin:
 - calcium channel modulation, 413–414
 - epilepsy efficacy, 417–418
- Galantamine:
 - dementia disorders and, 463–465
- γ -aminobutyric acid (GABA):
 - GABA_A receptors
 - antiepileptic drugs and, 411–412
 - felbamate, 414–415
 - topiramate, 415–416
 - sedatives:
 - benzodiazepine binding, 190–192
 - pharmacology, 180–181
 - structure, 178–180
 - narcolepsy therapy
 - modafinil, 101–103
 - suprachiasmatic nucleus and, 13–15
 - traumatic brain injury and, 451
- γ -hydroxybutyric acid (GHB):
 - cataplexy therapy, 112
 - disturbed nocturnal sleep therapy, 112
 - sleep paralysis and hypnagogic hallucination therapy, 112
- Gastrointestinal tract:
 - ghrelin modulation, 776
- Gavestinel:
 - stroke management, 359
- GBA polymorphisms:
 - idiopathic Parkinson's disease and, 543
- GBR12909:
 - narcolepsy therapy
 - dopaminergic neurotransmission, 94–95
- Genetic studies:
 - Tourette's syndrome, 266–267
- Genomic screening:
 - Tourette's syndrome, 267
- Ghrelin:
 - acylated peptide hormone, 766–768
 - adipocyte and body composition modulation, 774
 - agonists/antagonists, 777
 - antiproliferative/proliferative effect, 776
 - appetite and metabolic regulation, 772–773
 - bone effects, 776
 - cardiovascular action, 774–775
 - expression pattern, 768–769
 - gastrointestinal tract function, 776
 - growth hormone secretagogues, 769
 - growth hormone secretion, 773–774
 - immune system modulation, 775
 - pancreas function and insulin resistance, 775–776
 - peripheral function, 774–776
 - receptor
 - constitutive activity, 770–771
 - homologous subfamily, 771–772
 - receptor ligands, 766–769
 - research history on, 766
 - sympathetic nervous system, 774
 - thyroid function, 775
- Glatiramer acetate:
 - multiple sclerosis therapy, 676
- Glial cells:
 - human immunodeficiency virus excitotoxicity cell death, 706
 - myelin-forming, 592–593
 - neurotrophins and, 239
- Glial-derived neurotrophic factor (GDNF):
 - amyotrophic lateral sclerosis and, 226–227
 - discovery of, 221–223
 - future research on, 232–233
 - neuroinflammatory mechanisms, 641
 - neuronal maturation regulation, 224
 - pain perception and management, 229
 - Parkinson's disease and, 228–229, 505
- Glial transporters:
 - stroke management, 365–366
- Glucagon-like peptide 1 (GLP1):
 - leptin signaling and feeding regulation, 743
- Glucose:
 - leptin effect on, 746–747
- Glutamate neurotransmitters:
 - neurogenesis and, 207–208
 - Parkinson's disease treatment, 504–505
 - stroke and
 - ischemic brain injury, 351–353
 - NMDA receptor antagonists, 354–355
 - release inhibitors, 366–367

- Tourette's syndrome, 272
- traumatic brain injury and, 449–450
- Glutamatergic receptors:
 - autism spectrum disorders
 - medications, 331–332
 - dementia disorders and, 469–470
 - human immunodeficiency virus and
 - excitotoxicity cell death, 705–706
 - stroke pathophysiology and, 354, 355, 361–366
 - AMPA receptor antagonists, 361–363
 - glutamate transporters, 365–366
 - kainate receptor agonists, 363
 - metabotropic glutamate receptors, 363–365
- Glutamate transporters:
 - stroke management, 365–366
- Glyceroneogenesis:
 - lipolysis and thermogenesis, 799–801
- Glycine site antagonists, NMDA receptor:
 - stroke management, 357–359
- Glycolipids:
 - human neuropathy targeting, 614
- Glycoproteins:
 - human immunodeficiency virus, 699, 701
 - human neuropathy targeting, 614
 - in myelin, 604–607
- GM6001 inhibitor:
 - neuroinflammation and, 633
- Gonadotropin-releasing hormone (GHRH):
 - ghrelin effects and, 774
- Gp41 protein:
 - human immunodeficiency virus, 699, 701
- Gp120 protein:
 - human immunodeficiency virus, 699, 701
 - alcohol abuse and, 716
 - excitotoxicity cell death, 705–706
 - nigrostriatal system and, 709–710
- Gp130 cytokine receptor subunit:
 - inflammation mechanisms, 640
- G-protein-coupled receptors (GPCR):
 - hypocretin/orexin system, 126–127
 - receptors, 128
 - melatonin receptor molecular structure, 41–44
- G proteins:
 - ghrelin receptor activity, 770
- Green fluorescent protein (GFP):
 - invertebrate neurodegenerative disease
 - models
 - Parkinson's disease cell death, 570–577
- Growth factors:
 - amyotrophic lateral sclerosis and, 226–227
 - inflammation mechanisms, 639–641
 - neurogenesis and, 210–211
 - neurotrophin structure and, 240
 - stroke therapy and, 375–377
- Growth hormone secretagogues (GHS):
 - classification of, 766
 - ghrelin receptor evaluation, 770
 - ghrelin receptor ligands and, 769
- Growth hormone secretion:
 - ghrelin effects and, 773–774
- GTS-21 nicotinic agonist:
 - dementia disorders and, 468–469
- Guanfacine:
 - attention-deficit hyperactivity disorder
 - therapy, 299–300
 - autism spectrum disorders, 330–331
- Guillain-Barré syndrome:
 - myelin pathology, 613–614
- GYKI 52466 antagonist:
 - stroke management, 362
- Habit reversal training:
 - Tourette's syndrome therapy, 277–278
- Hallucinations:
 - hypnagogic hallucinations
 - symptoms of, 83
 - treatment of, 112
 - hypnopompic hallucinations
 - symptoms of, 83
 - Parkinson's disease and, 502–503
- Haloperidol:
 - autism spectrum disorders, 325
 - Tourette's syndrome and, 273–276
- HCRT gene:
 - hypocretin/orexin system, 127
- HCRT2 gene:
 - hypocretin/orexin system
 - narcolepsy and, 137–138
- Hematopoiesis:
 - prokineticins and, 170–171
- Hemizyosity:
 - parkin* gene mutations, familial
 - Parkinsonism, 531–532
 - PINK1 gene mutation and, 533–535
- High-density lipoprotein (HDL):
 - antiobesity therapy
 - rimonabant and, 827–828
- Highly active antiretroviral therapy (HAART):

- HIV neuropharmacology and, 694–695
 - methamphetamine/cocaine abuse and, 714–715
 - neurodegenerative disease and, 716–718
- High-throughput screening:
 - neurodegenerative disease, invertebrate models, 582–583
- Hippocampus:
 - hypocretin/orexin system
 - plasticity of, 136
 - neuronal survival mechanisms, 205–206
- Histamine:
 - hypocretin/orexin system
 - arousal mechanisms, 142–143
 - narcolepsy therapy, 105–106
- Histidine residues:
 - sedative/hypnotic binding, 190–192
- HIV-associated dementia (HAD):
 - alcohol abuse and, 716
 - apoptosis mechanisms, 704–705
 - classification, 694–695
 - epidemiology and pathogenesis, 695–696
 - lipid metabolism and, 707
 - methamphetamine/cocaine abuse and, 713–715
 - neurodegenerative disease and, 716–718
 - neurotransmitters and, 711–712
 - nigrostriatal system and, 708–710
 - Vpu protein and, 704
- Homologs:
 - ghrelin receptors, 771–772
 - p75 neurotrophin receptor structure, 241
- Homovanillic acid (HVA):
 - Parkinson's disease
 - neurochemistry, 481–483
- Hormones:
 - neuroinflammation and, 645
- Human autoimmune neuropathies, 613–614
 - animal models, 615
- Human immunodeficiency virus (HIV):
 - alcohol abuse and, 716
 - cholinergic system, 711–712
 - clinical features, 694–695
 - dementia and, 711–712
 - drug abuse and, 712–716
 - methamphetamine/cocaine, 713–715
 - opioid drugs, 715–716
 - excitatory amino acid neurotransmitters, 711
 - future neuropharmacology issues, 718
 - lipid metabolism alterations, 707
 - neurodegenerative diseases and, 716–718
 - neuropathology, 695–704
 - cell death cascades, 704–707
 - apoptosis, 704–705
 - excitotoxicity, 705–706
 - neural progenitor cells, 706–707
 - oxidative stress, 706
 - chemokines in, 697–698
 - neurodegenerative mechanisms, 699
 - neuropharmacology
 - features of, 694–695
 - future research issues, 718
 - neurotoxic proteins, 699–704
 - glycoproteins gp120 and gp41, 699, 701
 - Nef protein, 703
 - neurobiology of, 700
 - Rev protein, 704
 - Tat protein, 701–703
 - Vpr protein, 703
 - Vpu protein, 704
 - nigrostriatal system, 707–711
 - dopamine mediators, 709–711
 - pathogenesis, 695
- Human leukocyte antigen (HLA):
 - narcolepsy evaluation, 84
 - narcolepsy pathophysiology
 - immune system and, 85–86
 - neuroinflammation and, 626
- Huntington's disease:
 - invertebrate models of trinucleotide repeats, 577–579
- 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA):
 - multiple sclerosis therapy, 687
- Hydroxyindole-*O*-methyl transferase (HIOMT):
 - melatonin production, 40–41
- Hyperserotonin hypothesis:
 - autism spectrum disorders, 321–322
- Hyperzine A:
 - dementia disorders and, 463
- Hypnagogic hallucinations:
 - symptoms of, 83
 - treatment of, 112
- Hypnopompic hallucinations:
 - symptoms of, 83
- Hypnotics:
 - barbiturate-like drugs, 187
 - barbiturates, 184–187
 - basic principles, 177–178
 - benzodiazepines, 187–188
 - brain sites of action, 181–184

- GABA_A receptors
 - pharmacology, 180–181
 - structure, 178–180
- mouse studies, 190–192
- new agents, 188–190
- non-GABA_A receptor agents, 188
- safe development of, 184–190
- Hypocellularity:
 - adipocyte proliferative capacity, 797–799
- Hypocretin-1:
 - cataplexy therapy, 113–114
 - narcolepsy evaluation
 - in cerebrospinal fluid, 84
 - narcolepsy pathophysiology
 - deficiency in, animal models, 86
 - narcolepsy therapy
 - future research issues, 105–106
- Hypocretin-2:
 - history of, 127
- Hypocretin/orexin system:
 - afferents, 130
 - agonists/antagonists, 128
 - arousal, feeding behavior and motivation
 - integration, 144–145
 - arousal circuitry, 139–144
 - cholinergic systems, 143–144
 - dopaminergic systems, 143
 - histaminergic systems, 142–143
 - lateral hypothalamic neurons, 140–141
 - noradrenergic systems, 141–142
 - serotonergic systems, 142
 - autonomic endocrine effects, 134–135
 - excitatory neurotransmitters, 131–132
 - feeding and metabolism, 132–134
 - fiber projections, 129–130
 - hippocampal plasticity, 136
 - hypocretin cell bodies, 128–131
 - hypocretin discovery, 126–127
 - lateral hypothalamus, 125–126
 - motivation and addiction, 135
 - narcolepsy pathophysiology, 136–139
 - sleep regulation and, 86–89
 - pain and anesthesia, 136
 - receptors, 128
 - distribution of, 130–131
 - at synapses, 130
- Hypothalamic pathway:
 - sedative/hypnotic action sites, 182–184
- Hypothalamic-pituitary-adrenal (HPA) axis:
 - circadian rhythms and release of, 19–20
- Hypothalamus:
 - circadian rhythms and, 23–24
 - leptin signaling and and feeding
 - regulation, 741–743
- Idiopathic hypersomnia:
 - classification of, 84
 - modafinil therapy, 100–103
- Idiopathic Parkinson's disease (IPD):
 - genetics and, 542–543
 - parkin* gene mutations, 531–532
- IDRA 21 receptor modulator:
 - dementia disorders and, 470
- Ifenprodil:
 - stroke management, 357
- IGF binding proteins (IGFBPs):
 - stroke and, 376–377
- Immediate-release stimulants:
 - attention-deficit hyperactivity disorder
 - therapy, 294
- Immune system:
 - ghrelin modulation, 775
 - narcolepsy pathophysiology and, 85–86
- Immunization techniques:
 - neuroinflammation modulation, 644–645
- Immunomodulatory therapy:
 - multiple sclerosis, 674–676
 - novel drug development, 687–688
- Immunoreceptors:
 - inflammation and, 631–632
- Indoleamine 2,3-deoxygenase (IDO):
 - inflammation mechanisms, 625
 - tryptophan metabolism and, 644
- Infection:
 - Tourette's syndrome, 268–269
- Inflammation. *See also* Neuroinflammation
 - basic principles of, 622–623
 - brain immunoreceptors, 631–632
 - central nervous system response, 623–624
 - blood-brain barrier, 624–625
 - costimulatory molecules, 631
 - major histocompatibility classes, 631
 - neuroinflammation
 - antigen-presenting cells, 626
 - arachidonic acid pathways, 630
 - astrocytes, 627
 - blood-derived inflammatory cells, 628
 - chemokines, 629–630
 - cytokines, 628–629
 - endothelial cells, 627
 - growth factors, 639–641
 - humoral components, 628–630
 - immunization approaches, 644–645
 - microglia, 626–627

- neurons, 628
- pharmacological modification, 645–646
- radical formation and oxidative damage, 630
- pharmacology, 632–646
 - adenosine pathway purinergic receptor modulation, 643–644
 - antioxidants, 636–637
 - apoptosis modulation, 637–638
 - arachidonic acid/prostaglandin pathways, 634–635
 - cAMP/cGMP modulation, 635–636
 - cannabinoid receptor modulation, 641–642
 - central nervous system migration, 633
 - chimokine-directed approaches, 633–634
 - estrogen/hormones, 645
 - future research issues, 646
 - indoleamine deoxygenase and tryptophan metabolism, 644
 - ion-channel approaches, 642
 - proinflammatory pathway modulation, 638–639
 - statins, 643
 - vitamin D derivatives, 642–643
- toll-like receptors, 631–632
- Inflammatory cascade:
 - traumatic brain injury and, 447–449
- Inflammatory molecules:
 - circadian rhythms and, 21
- Inflammatory pathways:
 - stroke management, 368
- Insulin:
 - ghrelin modulation, 775–776
 - leptin and, 745–746
- Insulin-like growth factor-1 (IGF-1):
 - amyotrophic lateral sclerosis, 226–227
 - ghrelin modulation, 776
 - inflammation mechanisms, 640–641
 - stroke and, 376–377
- Integrins:
 - Tat protein binding, HIV immunodeficiency and, 702–703
- Intercellular adhesion molecule-1 (ICAM-1):
 - inflammation mechanisms, 624–625
 - statin therapy and, 643
 - stroke management, 369–370
- Interference RNA (RNAi):
 - Huntingdon's disease trinucleotide repeats invertebrate model, 577–579
 - Parkinson's disease invertebrate models, 573–577
- Interferon β 1b:
 - multiple sclerosis therapy, 674–675
- Invertebrate models:
 - neurodegenerative disease
 - Alzheimer's disease, 579–582
 - Caenorhabditis elegans* system, 568–569
 - Drosophila melanogaster*, 569
 - early research, 567–568
 - future applications, 582–583
 - genetic and molecular pathways, 568
 - Huntington's disease trinucleotide repeats, 577–579
 - Parkinson's disease:
 - dopamine neuron cell death, 569–577
- 2-[¹²⁵I]Iodomelatonin:
 - development of, 39–40
- Ion channels:
 - circadian rhythms and, 9
 - myelin pronodal proteins and, 609
 - neuroinflammation and, 642
- Ionotropic glutamate receptors:
 - antiepileptic drugs and, 413
- Janus kinase (Jak) gene:
 - leptin secretion and, 737–739
- Jet lag:
 - circadian rhythms, 15
- Jun-N-terminal kinase (JNK):
 - p75 neurotrophin receptor signaling, 246–247
 - Parkinson's disease and, 228–229
- JWS-USC-751X:
 - dementia disorders therapy and, 471–473
- K252a inhibitor:
 - tyrosine kinase receptor blockade, 232
- Kainate receptors:
 - antiepileptic drugs and topiramate, 416–417
 - neurogenesis and, 208
 - stroke and
 - antagonists, 363
 - ischemic brain injury, 351–353
- Ketanserin:
 - narcolepsy therapy
 - GABA inhibition, 101–102
- Kufor Rakeb disease (KRD):
 - familial Parkinson's disease and, 541

- Lamotrigine:
autism spectrum disorders, 332
epilepsy efficacy, 417–418
for secondary generalized epilepsy, 424
stroke management, 367
- Lateral hypothalamus (LH):
hypocretin/orexin system, 125–126
cell bodies, 128–129
fiber projections, 129–130
self-stimulation (LHSS), 135
- Lazaroids:
stroke management, 371–372
- Learning-related survival mechanisms:
neurogenesis, 204–206
- Lennox-Gastaut syndrome:
felbamate therapy, 414–415, 418
therapy options for, 421, 424
- Leptin:
early research, 733–734
energy expenditure and, 743–744
food intake regulation, 740–743
OB gene expression and, 734–736
peripheral nutrient utilization, 744–748
receptor expression, 736–739
signaling inhibition and resistance,
739–740
- Leucine-rich repeat kinase 2 (LRRK2) gene:
familial Parkinson's disease and mutation
of, 536–539
- Levetiracetam:
drug-resistant epilepsy, 425–426
synaptic vesicle protein SV2A
modulation, 414
- Levoamphetamine:
attention-deficit hyperactivity disorder
therapy, 297–298
- Levodopa:
human immunodeficiency virus
nigrostriatal system and, 710
Parkinson's disease treatment
peripheral decarboxylase inhibitor with,
484–487
slow-release formulations, 487–488
- Lewy bodies:
familial Parkinsonism and
PARK1 and PARK4 mutations,
527–529
Parkinson's disease
invertebrate models of cell death,
570–577
- Lifestyle factors:
idiopathic Parkinson's disease and, 544
- Light-dark cycles:
circadian rhythms, 5
- Lipid metabolism:
human immunodeficiency virus and, 707
leptin effect on, 746–747
- Lipolysis:
adipose tissue signaling and, 802–803
glyceroneogenesis, 799–801
leptin effect on, 747–748
- Lipostatic hypothesis, 733–734
- Lisuride:
Parkinson's disease treatment, 491
- Lithium:
circadian rhythms and, 15–17
- Long-term potentiation (LTP):
brain-derived neurotrophic factor
synthesis and, 224–225
glutamate receptors and, 470
hypocretin/orexin system, 136
- Lubeluzole:
stroke management, 367
- Luteinizing hormone (LH):
hypocretin/orexin system, 134–135
- Luzindole:
melatonin receptor inhibition, 39
in suprachiasmatic nucleus, 49–50
melatonin receptor molecular
pharmacology, 43–44
- LY377770 antagonist:
stroke and
kainate receptors, 363
- MAdCAM-1:
inflammation mechanisms, 624–625
- Madopar:
Parkinson's disease treatment, 484–487
slow-release formulations, 487–488
- Magnetoencephalography (MEG):
epilepsy diagnosis, 407
- Major histocompatibility complex (MHC):
neuroinflammation and, 626–627
class I/II immunoreceptors, 631
- Matrix metalloproteinases (MMPs):
neuroinflammation and, 633
minocycline inhibition, 645–656
Tat protein promotion of, 703
- Mazindol:
narcolepsy therapy, 103
- MC4 receptor agonist:
antiobesity therapy, 828–829
- MCH peptide:
hypocretin/orexin system, 129

- MCH receptor-1 antagonists:
 antiobesity therapy, 829–830
- Melanin-concentrating hormone (MCH):
 leptin signaling and and feeding
 regulation, 743
- Melanocortin pathways:
 leptin signaling and feeding regulation,
 741–743
- Melatonin receptors:
 central nervous system
 circadian rhythm modulation, 54–57
 clock genes and, 50–51
 desensitization, 52–53
 historical perspective, 38–40
 melatonin production, 40–41
 molecular pharmacology, 42–44
 molecular structure, 41–42
 overview, 37–38
 regulatory mechanisms, 51–54
 signaling mechanisms, 44–46
 supersensitization, 54
 suprachiasmatic nucleus, 46–54
 circadian inputs and outputs, 46–47
 receptor localization, signaling, and
 function, 47–50
 as therapeutic targets, 57–61
 circadian rhythms, 58
 depression, 58–61
 sleep, 57–58
 circadian rhythm modulation, 54–57
 desensitization function, 52–53
 regulatory mechanisms, 51–54
 sedative/hypnotics and, 188
 supersensitization function, 54
 in suprachiasmatic nucleus
 localization, signaling, and function,
 47–50
 therapeutic targeting of, 57–61
 circadian rhythms, 58
 depression, 58–61
 sleep, 57–58
 white adipose tissue innervation, 794–797
- Memantine:
 dementia disorders and, 470
- Membrane activity:
 circadian rhythms, 12
- Memory. *See also* Dementia
 brain-derived neurotrophic factor
 synthesis and, 224–225
 traumatic brain injury and, 446
- Mesial temporal sclerosis (MTS):
 epilepsy and, 405
- Messenger RNA (mRNA):
 hypocretin receptor distribution, 130–131
- Metabolism kinetics:
 ghrelin effects, 772–773
 hypocretin/orexin system, 132–134
 leptin gene, 733–734
- Metabotropic glutamate receptors:
 neurogenesis and, 208
 stroke management, 363–365
- Methamphetamine:
 HIV/AIDS and abuse of, 713–715
- 1-Methyl-4-phenyl-1,2,3,6-tetrahydro-
 pyridine (MPTP):
parkin gene mutations and, 532
 Parkinson's disease pathogenesis and,
 546
- Methylphenidate:
 attention-deficit hyperactivity disorder,
 294–296
 autism spectrum disorders, 329–330
 narcolepsy therapy, 90–93
- Methylprednisolone:
 traumatic brain injury and, 448–449
- Microglia:
 neuroinflammation and, 626–627
- Middle cerebral artery occlusion (MCAO):
 stroke-related global and focal ischemia,
 353–354
- Mild cognitive impairment:
 diagnostic criteria, 462
- Mild cognitive motor disorder (MCMD):
 HIV neuropharmacology and, 694–696
- Minocycline:
 multiple sclerosis therapy, 679
 neuroinflammation therapy with, 645–646
- Mirtazapine:
 autism spectrum disorders, 331, 334
- Missense mutations:
 familial Parkinson's disease and
 PINK1 gene mutation and, 534–535
- Mitochondrial DNA:
 idiopathic Parkinson's disease and, 543
- Mitochondrial dysfunction:
 Parkinson's disease pathogenesis and,
 545–547
- Mitogen-activated protein kinase (MAPK):
 adipose tissue signaling and, 802–803
- Mitoxantrone:
 multiple sclerosis therapy, 676–677
- MK-801 antagonist:
 human immunodeficiency virus
 nigrostriatal system and, 709

- stroke and NMDA receptors, 355–356, 361
- Modafinil:
 - attention-deficit hyperactivity disorder therapy, 301
 - narcolepsy therapy, 100–103
 - limits of, 114
 - Parkinson-related sleep disturbance, 504
- Molecular feedback loops:
 - circadian rhythms
 - animal models, 11–12
- Molecular genetics:
 - rhythmicity
 - Drosophila* models, 7
- Molecular targeting:
 - narcolepsy therapy
 - amphetamines, 93–94
- Monoamine neurotransmitters:
 - attention-deficit hyperactivity disorder therapy, 297–298
 - autism spectrum disorders, 331
 - narcolepsy pathophysiology
 - hypocretin/orexin system, 87–89
- Monoamine oxidase inhibitors (MAOIs):
 - cataplexy therapy, 111, 114–115
 - circadian rhythms and
 - antidepressant effects, 19–20
 - Parkinson's disease
 - neurochemistry of, 481–483
 - type B inhibitor (MAO_B), 494–496
- Monoclonal antibodies:
 - multiple sclerosis therapy, 677–679
 - new drug development, 684–687
- Monogenetic Parkinsonism:
 - causative mutations, 527–539
 - genetic mutations in, 525–541
 - non-PARK mutations, 540
 - PARK1 loci, 527–529
 - PARK2 (parkin) mutations, 529–532
 - PARK3 mutations, 541
 - PARK4 loci, 527–529
 - PARK5 (UCH-L1) mutation, 539–540
 - PARK6 (PINK1) mutations, 532–535
 - PARK7 (DJ-1) mutations, 535–536
 - PARK8 (LRRK2) mutation, 536–539
 - PARK9 mutations, 541
 - PARK10 mutations, 541
 - PARK11 mutations, 541
 - Parkinsonism loci, 540–541
 - PARK loci, 526–527
 - potential causative mutations, 539–541
- Monosynaptic H reflex:
 - narcolepsy pathophysiology, 85
- Monotherapies:
 - antiepileptic drugs, 419–420
- Motilin receptor:
 - ghrelin and, 771–772
- Motivation response:
 - hypocretin/orexin system, 135
 - arousal and feeding behavior
 - integration, 144
- Motor fluctuation:
 - Parkinson's disease treatment and classification of, 496–499
 - levodopa agents, 487
- Motor hyperactivity/inattention:
 - autism spectrum disorder, 333–334
- Mouse models:
 - circadian rhythms, 4–5
 - rhythmicity in, 8
 - sedative/hypnotics, 190–192
- Multidrug resistance:
 - drug-resistant epilepsy, 425–426
- Multidrug transporters:
 - drug-resistant epilepsy, 425–426
- Multifocal motor neuropathy (MMN):
 - characteristics of, 614–615
- Multinucleated giant cells (MNGCs):
 - HIV neuropathogenesis and, 697
- Multiple sclerosis (MS):
 - acute relapses
 - treatment of, 672–674
 - chemotherapy agents, 676–677
 - classification of, 671–672
 - immunomodulatory therapies, 674–676
 - monoclonal antibody therapy, 677–678
 - new drug development, 684–687
 - myelin pathology and, 612–613
 - new drug development, 678–679
 - immunomodulator/suppressants, 687–689
 - overview, 683–684
- Muscarinic receptor agonists:
 - dementia disorders and, 465–466
- Myelin:
 - CD9 protein in, 608
 - claudin protein family in, 608
 - CNS proteins, 596–599
 - composition, 592–609
 - lipids, 594–596
 - connexins in, 609
 - 2',3'-cyclic nucleotide
 - 3'-phosphodiesterase, 603–604

- disorders, 611–615
 - autoimmune demyelinating diseases, 611–615
 - axon-glia disruptions, 611
 - experimental allergic encephalomyelitis, 611–612
 - multiple sclerosis, 612–613
- enzymes in, 609–610
- future research issues, 615–616
- glycoproteins in, 604–607
- myelin and lymphocyte protein and plasmolipin, 608
- myelin basic protein, 598–599, 602–603
- paranodal proteins, 609
- peripheral myelin protein-22, 601–602
- PNS proteins, 599–603
- protein 2, 603
- protein locations in, 600
- protein zero in, 599–601
- proteolipid protein, 596–598
- receptors, 610
- structure of, 592
 - molecular organization, 592, 595
 - sheath structures, 592, 594
- tetraspan proteins in, 607–608
- Myelin and lymphocyte protein (MAL):
 - structure and function, 608
- Myelin-associated glycoprotein (MAG):
 - multiple sclerosis and, 613
 - stroke therapy, 377–378
 - structure and function, 604–606
- Myelin basic protein (MBP):
 - peripheral form, 602–603
 - structure and function, 596–598
- Myelin oligodendrocyte glycoprotein (MOG):
 - multiple sclerosis and, 613
 - structure and function, 606–607
- Narcolepsy:
 - evaluation, 83–84
 - cerebrospinal fluid hypocretin-1 assessment, 84
 - histocompatibility human leukocyte antigen testing, 84
 - polysomnography, 83
 - future research issues, 114–115
 - idiopathic hypersomnia, 84
 - overview of, 80
 - pathophysiology, 84–89
 - human leukocyte antigen analysis, 85–86
 - hypocretin/orexin system, 136–138
 - hypocretin/orexin system and sleep regulation, 86–89
 - hypocretin transmission:
 - animal models, 86
 - symptoms analysis, 84–85
- symptoms of, 80–83
 - cataplexy, 83
 - excessive daytime sleepiness, 82, 84, 90–92
- hypnagogic/hypnopompic
 - hallucinations, 83
- sleep paralysis, 83
- treatment of, 90–100
 - amphetamines, 90–100
 - drug interactions with, 98, 100
 - molecular targeting of, 93–94
 - side effects, 98
 - bupropion, 103–105
 - caffeine, 104–105
 - dopamine neurotransmitters:
 - substrates, 97–98
 - dopamine neurotransmitters and EEG arousal, 94–97
 - future stimulant development, 105–106
 - mazindol, 103
 - modafinil, 100–103
 - monoamine oxidase inhibitor reduction of, 111
 - nonamphetamine stimulants, 99–105
 - selegiline, 104
- Natalizumab:
 - multiple sclerosis therapy, 677–679, 684–685
- Nausea and vomiting:
 - Parkinson's disease treatment and, 500
 - levodopa agents, 486–487
- NBI-31772 molecule:
 - stroke therapy, 376–377
- NBQX antagonist:
 - stroke and, 361–362
- Nerve growth factor (NGF):
 - cognitive function and, 228
 - discovery, 221–222
 - inflammation mechanisms, 641
 - neuronal maturation regulation, 224
 - neuropathies and, 227
 - neurotrophins and, 237–238
 - signaling mechanisms, 239
 - p75 neurotrophin receptor
 - signaling, 246–247
 - structure, 240–241

- pain perception and management, 229
- peripheral nervous system and, 225
- structure of, 240
- tyrosine kinase receptor structure and, 241–242
- Neural progenitor cells:
 - human immunodeficiency virus and, 706–707
- Neural regeneration:
 - traumatic brain injury and, 452–453
- Neurite outgrowth inhibitor (NoGo):
 - stroke therapy, 377–378
- Neuroactive steroids/neurosteroids:
 - traumatic brain injury and, 450–451
- Neuroanatomy:
 - Tourette's syndrome, 269–270
- Neurochemistry:
 - dementia disorders and, 462–463
 - Parkinson's disease, 481–483
 - Tourette's syndrome, 270–273
 - white adipose tissue innervation, 794–797
- Neurodegenerative disease:
 - extra/intracellular protein aggregates and, 644–645
 - HIV neuropathogenesis and, 699, 716–718
 - inflammation mechanisms in, 622–623
 - invertebrate models
 - Alzheimer's disease, 579–582
 - Caenorhabditis elegans* system, 568–569
 - Drosophila melanogaster*, 569
 - early research, 567–568
 - future applications, 582–583
 - genetic and molecular pathways, 568
 - Huntington's disease trinucleotide repeats, 577–579
 - Parkinson's disease:
 - dopamine neuron cell death, 569–577
- Neurogenesis:
 - neurotrophin regulation of, 224
 - prokineticins and, 171–172
 - regulation in adults
 - future research issues, 211–212
 - multiple regulation points, 204
 - overview, 203–204
 - proliferation, 206–211
 - survival, 204–206
- Neuroimaging studies:
 - epilepsy diagnosis, 407
 - stroke management, 360–361
- Neuroinflammation:
 - antigen-presenting cells, 626
 - arachidonic acid pathways, 630
 - astrocytes, 627
 - blood-derived inflammatory cells, 628
 - chemokines, 629–630
 - cytokines, 628–629
 - endothelial cells, 627
 - growth factors, 639–641
 - humoral components, 628–630
 - immunization approaches, 644–645
 - microglia, 626–627
 - neurons, 628
 - pharmacological modification, 645–646
 - radical formation and oxidative damage, 630
 - stroke and
 - ischemic brain injury, 351–353
- Neuroleptics:
 - Tourette's syndrome and, 273–276
- Neuronal organization:
 - cognitive function and diversity in, 461–463
 - developmental maturation, 224
 - modulation in adults, 224–225
 - narcolepsy therapy, dopaminergic effects, 97–98
 - neurogenesis
 - survival mechanisms, 204–206
 - neuroinflammation, 628
 - neurotrophins and, 239
 - survival and differentiation during development, 222–223
- Neuropathy:
 - human autoimmune neuropathies, 613–614
- Neuropeptides:
 - neurogenesis and, 210–211
- Neuropeptide Y (NPY):
 - ghrelin effects and, 773
 - hypocretin/orexin system and, 132–134
 - leptin signaling and feeding regulation, 742–743
 - suprachiasmatic nucleus and, 13–15
 - white adipose tissue innervation, 796–797
- Neuroplasticity:
 - traumatic brain injury and, 453–454
- Neuroprotective agents:
 - dementia disorders and, 469
 - Parkinson's disease and, 504–505
 - stroke management
 - antioxidants, 371–372
 - apoptosis and caspase inhibitors, 372–373
 - development criteria, 374

- downstream approaches, 367–371
 - glutamate release inhibitors, 366–367
 - metabotropic glutamate receptors, 364–365
- Tat protein, 702–703
- traumatic brain injury and, 446–451
 - anti-inflammatory agents, 447–449
 - free-radical scavengers, 447
 - neuroactive steroids/neurosteroids, 450–451
 - neurotransmitter agonists/antagonists, 449–450
- Neurotoxic cascade:
 - traumatic brain injury and, 444–446
- Neurotoxicity:
 - human immunodeficiency virus neuropathogenesis:
 - chemokines and, 698–699
 - proteins, 699–704
 - glycoproteins gp120 and gp41, 699, 701
 - Nef protein, 703
 - neurobiology of, 700
 - Rev protein, 704
 - Tat protein, 701–703
 - Vpr protein, 703
 - Vpu protein, 704
- Parkinson's disease
 - invertebrate models of cell death, 570–577
- Neurotransmitters:
 - agonists/antagonists
 - traumatic brain injury and, 449–450
 - dementia disorders and, 462–463
 - human immunodeficiency virus dementia and, 711–712
 - in myelin, 610
 - Parkinson's disease
 - neurochemistry, 482–483
 - stroke therapy and modulation of, 377
- Neurotrophic factor 3 (NT-3):
 - discovery of, 221–223
 - neurogenesis regulation, 224
 - neuronal maturation regulation, 224
 - neuropathies and, 227
 - structure of, 240
 - in suprachiasmatic nucleus
 - melatonin receptor actions, 50
- Neurotrophic factor 4/5 (NT-4/5):
 - discovery of, 221–223, 238
 - obesity and weight control, 229–230
 - Parkinson's disease and, 228–229
 - structure of, 240
- Neurotrophic factors (NTFS):
 - adult neurogenesis, 224
 - drug discovery mechanisms, 230–232
 - biological antagonists, 231
 - direct receptor agonists/antagonists, 231–232
 - indirect modulators, 232
 - future research on, 232–233
 - history of, 221–222
 - HIV neuropathology
 - apoptosis and, 705
 - neuroinflammatory mechanisms, 641
 - neuronal function modulation, 224–225
 - neuronal maturation in development, 224
 - neuronal survival and differentiation, 222–224
 - physiological functions of, 222–225
 - therapeutic applications, 225–230
 - Alzheimer's disease and cognitive impairment, 227–228
 - amyotrophic lateral sclerosis, 225–227
 - depression, 229–230
 - obesity and weight control, 229–230
 - pain management, 229
 - Parkinson's disease, 228–229
 - peripheral neuropathies, 227
 - traumatic brain injury and, 452–453
- Neurotrophic hypothesis, 239
- Neurotrophin-3 (NT-3):
 - discovery of, 238
- Neurotrophins:
 - history of, 237–238
 - preference determinants, 242
 - receptors, 238
 - p75-TRK interactions, 247–248
 - signaling, 242–247
 - structure, 240–242
 - retrograde axonal signaling, 248–249
 - signaling functions, 238–239
 - structure, 239–240
- Neurturin (NRTN):
 - discovery of, 221–223
 - Parkinson's disease and, 228–229
- Nev protein:
 - HIV neurotoxicity, 703
- NF- κ B:
 - HIV neuropathology and, 705
- Nicotine:
 - dementia disorders and, 463–465

- Nicotine gum:
 - Tourette's syndrome, 277
- Nicotinic acetylcholine receptors:
 - dementia disorders and, 463–465
 - agonist therapies, 467–468
- Nicotinic receptor agonists:
 - dementia disorders and, 467–468
 - neuroprotective aspects of, 469
 - therapeutic targeting efficacy, 471–473
- Nigrostriatal system:
 - human immunodeficiency virus and, 707–710
- Nitric oxides:
 - neuroinflammation and, 630
 - pharmacology of, 636–637
 - parkin* gene mutations and, 532
 - traumatic brain injury and, 447
- Nitric oxide synthase (NOS) inhibitor:
 - stroke management, 370–371
- NMDA (*N*-methyl-D-aspartic acid) receptors:
 - antiepileptic drugs and
 - felbamate, 414–145
 - topiramate, 416–417
 - dementia disorders and, 470
 - human immunodeficiency virus and
 - alcohol abuse and, 716
 - excitotoxicity cell death, 705–706
 - neurogenesis and, 208
 - neuroinflammation and
 - antioxidant modulation, 637
 - stroke and
 - antagonist development, 354–358
 - side effects, 359–360
 - ischemic brain injury, 351–353
 - traumatic brain injury and, 449–450
- NNC 09-0026 compounds:
 - stroke management, 367
- Nocturnal sleep studies:
 - narcolepsy evaluation, 83
- Nonphotic stimuli:
 - melatonin receptors
 - clock gene expression, 51
- Noradrenergic agents:
 - attention-deficit hyperactivity disorder
 - therapy, 299–301
 - hypocretin/orexin system
 - arousal mechanisms, 141–142
- Norepinephrine:
 - adipocyte proliferative capacity, 798–799
 - narcolepsy therapy
 - amphetamine targeting, 92–94
 - adverse effects, 98
 - anatomical substrates, 97–98
 - drug interactions, 98, 100
 - EEG arousal and, 94–97
- Norepinephrine transporters:
 - attention-deficit hyperactivity disorder
 - therapy, 297–298
- NS-649 compound:
 - stroke management, 367
- Nurr1* gene:
 - familial Parkinson's disease and, 540
- Obesity and weight control:
 - adipose tissue
 - catecholamine signaling mechanisms, 801–803
 - cell types and depots, 786–789
 - future research issues, 804
 - glyceroneogenesis, 799–801
 - sympathetic nervous system
 - innervation, 789–797
 - adipocyte proliferative capacity, 797–799
 - anterograde tract-testing, SNS to WAT, 792–797
 - retrograde tracing neuroanatomical studies, 790–792
 - white vs. brown adipocytes, 787–789
- epidemiology, 815–816
- leptin genetics and
 - early research, 733–734
 - energy expenditure and, 743–744
 - food intake regulation, 740–743
 - OB* gene expression and, 734–736
 - peripheral nutrient utilization, 744–748
 - receptor expression, 736–739
 - signaling inhibition and resistance, 739–740
- neurotrophic factors and, 229–230
- pharmacotherapy, 816–821
 - axokine, 822–824
 - CCK_A receptor agonists, 830–831
 - future research issues, 831–833
 - historic perspectives, 817–818
 - MC4 receptor agonists, 828–829
 - MCH receptor-1 antagonists, 829–830
 - new drug development, 821–831
 - orlistat, 820–821
 - PYY3-36 peptide agonists, 831
 - rimonabant, 824–828
 - serotonin receptor 5-HT_{2C} agonists, 828
 - sibutramine, 818–820

- OB* gene:
 - leptin secretion and expression of, 734–736
- Obsessive-compulsive disorder (OCD):
 - Tourette's syndrome comorbidity, 266–268, 276–277
- Olanzapine:
 - autism spectrum disorders, 327–328
 - Tourette's syndrome therapy, 275–276
- Olfactory bulb granule cell precursors:
 - neurogenesis and, 211–212
- Oligodendrocyte myelin glycoprotein (OMgp):
 - structure and function, 607
- Opiates/opioids:
 - HIV neuropharmacology and, 715–716
 - Tourette's syndrome, 272–273
- Orlistat:
 - obesity therapy with, 820–821
- Orthostatic hypotension:
 - Parkinson's disease treatment and, 500
 - levodopa agents, 486–487
- Osteogenic protein-1 (OP-1):
 - stroke and, 376–377
- Output pathways:
 - suprachiasmatic nucleus, 12–13
- Ovarian disease:
 - prokineticins and, 170
- Oxidative stress:
 - familial Parkinson's disease and
 - DJ-1* gene mutation, 536
 - human immunodeficiency virus and, 706
 - methamphetamine/cocaine abuse and, 714–715
 - Parkinson's disease pathogenesis and, 545–547
- p38 mitogen-activated protein kinase (MAPK) pathway:
 - stroke management, 369
- p75 neurotrophin receptor:
 - discovery of, 238
 - functional interactions, 247–248
 - signaling mechanisms, 246–247
 - structure and function, 222–224, 240–241
 - synaptic function and, 239
- Painful dystonia:
 - Parkinson's disease treatment and, 499
- Pain perception and management:
 - hypocretin/orexin system, 136
 - neurotrophic factors and, 229
 - Parkinson's disease and, 504
 - prokineticins and, 166–167
- Pancreas function:
 - ghrelin modulation, 775–776
- Paranodal proteins:
 - structure and function, 609
- Parasympathetic nervous system:
 - white adipose tissue in, 792
- Parkin* gene:
 - familial Parkinsonism and
 - PARK2 mutations and, 529–532
 - idiopathic Parkinson's disease and, 542–543
 - invertebrate models, 576–577
- Parkinson's disease loci:
 - familial Parkinson's disease and, 540–541
- Parkinson's disease (PD):
 - classification, 524–525
 - etiology and classification, 480
 - familial (monogenetic) Parkinsonism, 525–541
 - causative mutations, 527–539
 - non-PARK mutations, 540
 - PARK1 loci, 527–529
 - PARK2 (*parkin*) mutations, 529–532
 - PARK3 mutations, 541
 - PARK4 loci, 527–529
 - PARK5 (*UCH-L1*) mutation, 539–540
 - PARK6 (*PINK1*) mutations, 532–535
 - PARK7 (*DJ-1*) mutations, 535–536
 - PARK8 (*LRRK2*) mutation, 536–539
 - PARK9 mutations, 541
 - PARK10 mutations, 541
 - PARK11 mutations, 541
 - Parkinsonism loci, 540–541
 - PARK loci, 526–527
 - potential causative mutations, 539–541
 - future treatment options, 505–506
 - in HIV/AIDS, 718
 - idiopathic Parkinsonism
 - classification, 524
 - environmental factors, 543–545
 - genetics, 542–543
 - invertebrate models
 - dopamine neuron cell death, 569–577
 - mitochondria and oxidative stress
 - pathogenesis linked to, 545–547
 - motor fluctuations and dyskinesia, 497–499
 - delayed "on," 498–499
 - diphasic dyskinesia, 499
 - dose failure, 499

- painful dystonia, 499
- peak-dose dyskinesia, 499
- sudden "off," 498
- wearing off phenomenon, 497–498
- yo-yo-ing, 499
- motor symptom treatments, 484–497
 - amantadine, 496–497
 - anticholinergics, 496
 - antiglutamate agents, 496–497
 - apomorphine, 493–494
 - bromocriptine, 488, 490
 - cabergoline, 491
 - COMT inhibitors, 495
 - dihydroergocryptine, 491
 - dopamine agonists, 488–494
 - dopaminomimetic agents, 484–496
 - entacapone, 496
 - levodopa plus peripheral decarboxylase inhibitor, 484–487
 - levodopa slow-release formulations, 487–488
 - lisuride, 491
 - monoamine oxidase type B inhibitor, 494
 - nondopaminomimetic agents, 496–497
 - pergolide, 490–491
 - piribedil, 493
 - pramipexole, 491–492
 - rasagiline, 495
 - ropinirole, 492
 - selegiline, 494–495
 - stalevo, 496
 - tolcapone, 495
- neurochemistry, 481–483
- neuroprotective therapy, 504–505
- neurotrophic factor therapy and, 228–229
- nonmotor symptoms treatment, 500–504
 - dementia, 502
 - depression and anxiety, 500–502
 - nausea and vomiting, 500
 - orthostatic hypotension, 500
 - psychosis and hallucinations, 502–503
- pharmacotherapy
 - current treatments, 483–484
 - history of, 480–481
- Paroxetine:
 - autism spectrum disorders, 324–325
 - melatonin receptor targeting, 60–61
- Pathogen-associated molecular patterns (PAMPs):
 - neuroinflammation and, 632
- PD 6735 compound:
 - melatonin receptor targeting, 58
- PD90780:
 - nerve growth factor inhibitor, 232
- Peak-dose dyskinesia:
 - Parkinson's disease treatment and, 499
- Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS):
 - Tourette's syndrome, 268–269
- Peptide YY:
 - antiobesity therapy
 - PYY3-36 peptide agonists, 831
 - white adipose tissue innervation, 796–797
- Per1* messenger RNA:
 - benzodiazepines, 20–21
- Pergolide:
 - Parkinson's disease and, 490–491
 - Tourette's syndrome therapy, 276
- Period genes:
 - melatonin receptors, 50–51
- Peripheral myelin protein-22:
 - structure and function, 601–602
- Peripheral neuropathies:
 - neurotrophic factor therapy, 227
- Peripheral nutrient utilization:
 - leptin and, 744–746
- Peroxisome proliferator-activated receptor gamma (PPAR γ):
 - neuroinflammation, 635
- Persephin (PSPN):
 - discovery of, 221–223
- Pervasive developmental disorders (PDDs):
 - aggression/self-injury, 334
 - anxiety and depression, 334
 - interfering repetitive behaviors, 334
 - motor hyperactivity and inattention, 333–334
 - overview, 320–321
 - pharmacology, 321–333
 - adrenergic agonists, 330
 - amantidine, 332
 - antiepileptic drugs, 332–333
 - antipsychotics, 325–328
 - atypical antipsychotics, 326
 - clomipramine, 322
 - clonidine, 330
 - clozapine, 326
 - d-cycloserine, 332
 - dopaminergic medications, 325–330
 - fluoxetine, 323–324

- fluvoxamine, 322–323
 - glutamatergic medications, 331–332
 - guanfacine, 330–331
 - haloperidol, 325
 - lamotrigine, 332
 - methylphenidate, 329–330
 - mirtazapine, 331
 - olanzapine, quetiapine, ziprasidone, and aripipazole, 327–328
 - pimozide, 325–326
 - risperidone, 326–327
 - serotonergic medications, 321–325
 - sertraline, citalopram, escitalopram, and paroxetine, 324–325
 - stimulants, 328–330
 - venlafaxine, 331
 - syndromes within, 333–335
- Pesticides:
 - idiopathic Parkinson's disease and, 545
- P-glycoprotein (P-gp):
 - drug-resistant epilepsy, 425–426
- Phen-Fen:
 - history of, 818
- Phenobarbital:
 - as antiepileptic drug, 418–419
 - focal/localization-related epilepsy therapy, 421, 423
- Phentermine:
 - as anti-obesity agent, 817–818
- α -Phenyl-*N*-*tert*-butylnitrone (PBN):
 - stroke management, 371–372
- Phenytoin:
 - development and testing, 419
 - epilepsy efficacy, 417–418
- Phosphoenolpyruvate carboxykinase (PEPCK):
 - leptin effect on, 746–747
- Phosphatase and tensin (PTEN)-induced putative kinase 1 (PINK1)
 - mutation:
 - familial Parkinson's disease and, 532–535
- Phosphatase and tensin (PTEN) pathway:
 - familial Parkinson's disease genetics and, 532–535
- Phosphodiesterases (PDEs):
 - neuroinflammation and, 635–636
- Phosphoenolpyruvate carboxykinase (PEPCK-C):
 - glyceroneogenesis control, lipolysis and thermogenesis, 800–801
- Phosphoinositol kinase (PI3):
 - tyrosine kinase receptor signal transduction, 243
- Phospholipase C γ -1:
 - tyrosine kinase receptor signal transduction, 242–243
- Photoc response:
 - melatonin receptors
 - clock gene expression, 51
 - suprachiasmatic nucleus and, 14–15
- Physostigmine:
 - dementia disorders and, 464–465
- Pimozide:
 - autism spectrum disorders, 325–326
- Pineal gland:
 - melatonin modulation of circadian rhythms, 55–57
- Piribendil:
 - Parkinson's disease therapy, 493
- Pituitary adenylate cyclase-activating peptide (PACAP):
 - neurogenesis and, 210–211
- PLA₂ enzymes:
 - neuroinflammation
 - arachidonic acid/prostaglandin pathways, 634–635
- Plasmolipin:
 - structure and function, 608
- Polyamine site antagonists, NMDA
 - receptor:
 - stroke and, 357
- Poly-Q repeats:
 - Huntington's disease, invertebrate models, 577–579
- Polysomnography:
 - narcolepsy evaluation, 83
- Potassium channels:
 - circadian rhythmicity and, 9
 - leptin and nutrient utilization, 745–746
 - neuroinflammation and, 642
- PPT-LDT pathway:
 - sedative/hypnotic action sites, 183–184
- Pramipexole:
 - Parkinson's disease treatment, 491–492, 502
- Prazosin:
 - narcolepsy therapy
 - GABA inhibition, 101–102
- Pregabalin:
 - calcium channel modulation, 413–414
 - epilepsy efficacy, 417–418

- Pregnancy:
 - antiepileptic drugs in, 424–425
- Presenilin:
 - Alzheimer's disease
 - invertebrate models, 580–582
- Primary progressive multiple sclerosis (PPMS):
 - classification, 671–672
- Progesterone:
 - traumatic brain injury and, 450–451
- Progressive multifocal leukoencephalitis (PML):
 - neuroinflammation and, 633
- Proinflammatory pathways:
 - neuroinflammation and
 - modulation of, 638–639
- Prokineticins:
 - angiogenesis and reproduction, 169–170
 - basic properties, 163, 165
 - circadian clock regulation, 167–168
 - feeding behavior, 168–169
 - future potential, 172
 - hematopoiesis, 170–171
 - neurogenesis and, 171–172
 - pain perception and management, 166–167
 - receptors, 164
 - smooth muscle contractility regulation, 166
 - structure and function, 164, 166
- Proliferation mechanisms:
 - neurogenesis and, 206–211
- Proneurotrophins:
 - structure, 239–240
- Pro-opiomelanocortin (POMC):
 - antiobesity therapy
 - axokine, 823–824
- Prostaglandin E-synthases (PGESs):
 - neuroinflammation, 635
- Prostaglandins:
 - neuroinflammation and, 630
 - pharmacological activation of, 634–635
- Protein 2:
 - structure and function, 603
- Protein chemistry:
 - GABA_A receptor structure
 - sedative/hypnotic mechanisms, 179–180
 - myelin, 594–609
- Protein kinase A:
 - adipose tissue signaling and, 802–803
- Protein tyrosine phosphatase 1B (PTP1B):
 - leptin signaling inhibition and resistance, 739–740
- Protein zero:
 - structure and function, 599–601
- Proteolipid protein (PLP):
 - structure and function, 596–598
- Pseudorabies virus (PSV):
 - white adipose tissue innervation, 792–797
- Psychosis:
 - Parkinson's disease and, 502–503
- Psychostimulants:
 - human immunodeficiency virus
 - nigrostriatal system and, 710
- Psychotropic drugs:
 - neurogenesis and, 210
- Purinergic receptors:
 - neuroinflammation
 - adenosine pathway and, 643–644
- QT prolongation:
 - Tourette's syndrome therapy, 274–276
- Quaaludes:
 - structure and function, 187
- Quetiapine:
 - autism spectrum disorders, 327–328
 - Tourette's syndrome therapy, 275–276
- Quinolinic acid:
 - inflammation and, 644
- Quinoxalinediones:
 - stroke management, 361–362
- Ramelteon:
 - melatonin receptor targeting, 57–58
- Rapid-eye-movement (REM) sleep:
 - antidepressants and, 17–18
 - ghrelin effects, 774
 - melatonin modulation of circadian rhythms, 56–57, 58
 - monoamine oxidase inhibitor reduction of, 111
 - narcolepsy pathophysiology, 84–85
 - hypocretin/orexin system, 89
- Reactive nitrogen species (RNS):
 - neuroinflammation and, 630
 - pharmacology of, 636–637
- Reactive oxygen species (ROS):
 - neuroinflammation and
 - pharmacology of, 636–637
 - Parkinson's disease pathogenesis and, 546–547
- Receptor agonists/antagonists:
 - ghrelin receptors, 777
 - neurotrophic factors as, 231–232
 - traumatic brain injury and, 449–450

- Relapsing remitting multiple sclerosis (RRMS):
 betaseron therapy, 674–675
 classification, 671–672
 multiple sclerosis therapy, 686
- Remacemide:
 stroke management, 358–359
- Repetitive behaviors:
 autism spectrum disorders, 334
- Reproductive function:
 prokinetics and, 169–170
- Retinohypothalamic tract (RHT):
 suprachiasmatic nucleus and, 13–15
- Retrograde axonal signaling:
 neurotrophins, 248–249
- Reverse genetics:
 Parkinson's disease
 invertebrate models, 573–577
- Rev protein:
 HIV neurotoxicity and, 704
- RhoA:
 p75 neurotrophin receptor, 247
- Rhythmicity:
 cellular basis, 8
Drosophila models, 7
- Riluzole:
 stroke management, 367
- Rimonabant:
 antiobesity therapy, 823–828
- Risk factors:
 idiopathic Parkinson's disease, 542–543
- Risperidone:
 attention-deficit hyperactivity disorder therapy, 301
 autism spectrum disorders, 326–327
 Tourette's syndrome therapy, 274–276
- Ritalin. *See* Methylphenidate
- Rituximab:
 multiple sclerosis therapy, 687
- RJR-2429 nicotinic agonist:
 dementia disorders and, 467–468
- Ropinirole:
 Parkinson's disease treatment, 492
- Rotenone exposure:
 Parkinson's disease and
 invertebrate models, 577
- Rotigotine:
 Parkinson's disease treatment, 505
- Running:
 neurogenesis and, 206
- S20304 agonist:
 melatonin receptors, therapeutic targeting of, 59–61
- Satiety signal:
 leptin and, 733–734
- SDZ EAA 494 antagonist:
 stroke management and, 356–357
- Secondary lymphoid tissue:
 neuroinflammation and formation of, 629
- Secondary progressive multiple sclerosis (SPMS):
 betaseron therapy and, 675
 classification, 671–672
- Second-messenger systems:
 Tourette's syndrome, 273
- Secretin:
 hypocretin/orexin system and, 127
- Sedatives:
 barbiturate-like drugs, 187
 barbiturates, 184–187
 basic principles, 177–178
 benzodiazepines, 187–188
 brain sites of action, 181–184
 GABA_A receptors
 pharmacology, 180–181
 structure, 178–180
 mouse studies, 190–192
 new agents, 188–190
 non-GABA_A receptor agents, 188
 safe development of, 184–190
- Seizure disorders:
 acute seizures, 427–428
 classification and definition, 404
 generalized seizures
 classification, 404–405
- Selective serotonin reuptake inhibitors (SSRIs):
 autism spectrum disorders, 322–325, 333–334
 melatonin receptors, therapeutic targeting of, 58–61
 Parkinson's disease and, 501
 sleep effects, 17–18
- Selegiline:
 cataplexy therapy, 111
 human immunodeficiency virus
 nigrostriatal system and, 710
 narcolepsy therapy, 104
- Self-injurious behaviors:
 autism spectrum disorders, 334
- Selfotel:
 stroke management, 358–359

- Serotonin (5-HT) neurotransmitters:
 - autism spectrum disorders, 321–325
 - circadian rhythms and
 - antidepressant effects, 18–20
 - hypocretin/orexin system
 - arousal mechanisms, 142
 - melatonin production, 40–41
 - neurogenesis and, 209–210
 - sedative/hypnotics and, 188
 - suprachiasmatic nucleus and, 14–15
 - Tourette's syndrome, 272
- Serotonin receptors:
 - melatonin receptors and, 38–39
 - serotonin receptor 5-HT_{2A}
 - sedative/hypnotics and, 190
 - serotonin receptor 5-HT_{2C} agonist
 - antiobesity therapy, 828
- Sertraline:
 - autism spectrum disorders, 324–325
- Serum response element (SRE) pathway:
 - ghrelin receptor activity, 770
- Sex steroids:
 - neurogenesis and, 207
- Shc gene:
 - tyrosine kinase receptor signal
 - transduction, 243
- SIB-1533A nicotinic agonist:
 - dementia disorders and, 467–468
- Sibutramine:
 - as obesity therapy, 818–820
- Sickness behavior:
 - circadian rhythms and
 - cytokines and, 21–23
- Signaling mechanisms:
 - leptin resistance and inhibition, 739–740
 - melatonin receptors, 44–46
 - neurotrophic factors, 224–225
 - neurotrophins, 238–239
 - tyrosine kinase receptors, 242–244
- Signal transducer and activator of transcription (STAT):
 - leptin receptor and expression, 737–739
 - leptin signaling inhibition and resistance, 739–740
- Sinemet:
 - Parkinson's disease treatment, 484–487
 - slow-release formulations, 487–488
- Single nucleotide polymorphism (SNP):
 - familial Parkinson's disease and
 - PINK1 gene mutation and, 534–535
- Sipatrigine:
 - stroke management, 367
- Site-directed mutagenesis:
 - melatonin receptor molecular structure, 42
 - neurotrophin structure and, 240
- Sjogren's syndrome:
 - AF102B agonist and, 466
- Sleep disorders:
 - antidepressants and, 17–18
 - cataplexy
 - defined, 82
 - narcolepsy pathophysiology, 85
 - human leukocyte antigen and, 85–86
 - pharmacological treatment:
 - future antiepileptics, 113–114
 - historical overview, 106–107
 - monoamine oxidase inhibitors, 111
 - neurotransmission, animal studies, 109–110
 - receptor subtypes, 110–111
 - second- and third-generation
 - antidepressants, 108
 - sodium oxybate, 112
 - tricyclic antidepressants, 107–109
 - circadian rhythms
 - chronopharmacology, 15–23
 - functional importance, 4
 - future research issues, 23–24
 - mammalian structural models, 10–13
 - nonmammalian structural models, 6–10
 - properties, 4–6
 - superchiasmatic nucleus period and
 - phase, 13–15
 - disturbed nocturnal sleep
 - treatment of, 112
 - melatonin receptors
 - therapeutic targeting, 57–58
 - narcolepsy
 - evaluation, 83–84
 - cerebrospinal fluid hypocretin-1
 - assessment, 84
 - histocompatibility human leukocyte antigen testing, 84
 - polysomnography, 83
 - future research issues, 114–115
 - idiopathic hypersomnia, 84
 - overview of, 80
 - pathophysiology, 84–89
 - human leukocyte antigen analysis, 85–86
 - hypocretin/orexin system and sleep regulation, 86–89

- hypocretin transmission
 - animal models, 86
 - symptoms analysis, 84–85
 - symptoms of, 80–83
 - cataplexy, 83
 - excessive daytime sleepiness, 82, 84, 90–92
 - hypnagogic/hypnopompic
 - hallucinations, 83
 - sleep paralysis, 83
 - treatment of, 90–100
 - amphetamines, 90–100
 - drug interactions with, 98, 100
 - molecular targeting of, 93–94
 - side effects, 98
 - bupropion, 103–105
 - caffeine, 104–105
 - dopamine neurotransmitters
 - substrates, 97–98
 - dopamine neurotransmitters and EEG arousal, 94–97
 - future stimulant development, 105–106
 - mazindol, 103
 - modafinil, 100–103
 - nonamphetamine stimulants, 99–105
 - selegiline, 104
 - Parkinson's disease and, 503
 - sleep paralysis
 - symptoms of, 83
 - treatment of, 112
- Sleep paralysis:
 - symptoms of, 83
 - treatment of, 112
- Small-molecular-weight peptide agonists:
 - cataplexy therapy, 115
- Smoking:
 - idiopathic Parkinson's disease and, 544
- Smooth muscle contractility:
 - prokineticin regulation, 166
- SNCA* gene mutation:
 - familial Parkinsonism and, 527–529
 - idiopathic Parkinson's disease and, 542–543
- Sodium bromide:
 - as antiepileptic drug, 418–419
- Sodium channels:
 - epilepsy and modulation of, 411
 - stroke management and modulation of, 367
- Sodium oxybate. *See* γ -hydroxybutyric acid (GHB)
 - cataplexy therapy, 112
- Sonic hedgehog (Shh) morphogen:
 - stroke therapy, 378
- SPD 502 antagonist:
 - stroke management, 362
- Splice variants:
 - leptin receptor expression and, 738–739
 - tyrosine kinase receptors, 245–246
- Statins:
 - multiple sclerosis therapy, 678–679
 - novel drug development, 687–688
 - neuroinflammation and, 643
- Status epilepticus, 427–428
- Stem cells:
 - stroke therapy, 378–379
 - traumatic brain injury and, 452–453
- Steroids:
 - stroke management, 371–372
 - traumatic brain injury and, 450–451
- Sterol regulatory element binding protein (SREBP1):
 - leptin effect on, 747–748
- Stimulant therapy:
 - attention-deficit hyperactivity disorder, 293–298
 - adverse effects, 298
 - amphetamines, 296–298
 - dexmethylphenidate, 296
 - extended-release methylphenidate, 295
 - immediate-release agents, 294
 - methylphenidate hydrochloride, 294–296
 - autism spectrum disorders, 328–330
- Stress:
 - neurogenesis and, 206–207
- Stroke:
 - AMPA receptor antagonists, 361–363
 - amphetamine and neurotransmitter
 - modulators, 377
 - antiadhesion molecules, 369–370
 - anti-nogo (IN-1) inhibitors, 377–378
 - antioxidants, 371–372
 - apoptosis and caspase inhibitors, 372–373
 - basic characteristics and symptoms, 348–349
 - brain injury with
 - classification, 350
 - mechanisms of, 351–353
 - repair approaches, 377–379
 - calcium channel blockers, 366–367
 - chemokine inhibition, 369
 - cytokine inhibition, 368–369
 - decahydroisoguinolines, 362

- down-stream approaches, 367–368
- erythropoietin, 377
- global/focal ischemia
 - animal models, 353–354
- glutamate/glutamatergic receptors, 354–355, 361–366
- glutamate release inhibitors, 366–367
- glutamate transporters, 365–366
- growth factors, 375–377
- GYKI 52466-related benzodiazepines, 362
- inflammatory pathways, 368
- kainate receptor antagonists, 363
- metabotropic glutamate receptors, 363–365
- NBQX-related quinoxalinediones, 361–362
- neuroprotective techniques, 366–373
 - development criteria, 374
- neurotrophic factors and, 229–230
- nitric oxide synthase inhibition, 370–371
- NMDA receptor antagonists, 354–359
 - clinical trial data, 358–359
 - competitive/noncompetitive agonists, 356–357
 - glycine site antagonists, 357–358
 - MK-801 compound, 355–356
 - polyamine site antagonists, 357
 - side effects, 359–360
- p38 inhibition, 369
- prevalence and incidence, 349–350
- sodium channel blockers, 367
- sonic hedgehog approach, 378
- stem cell approach, 378–379
- time window issues, 360–361
- upstream techniques, 366–373
- Substantia nigra pars compacta (SNc):
 - Parkinson's disease neurochemistry and, 481–483
 - invertebrate models of cell death, 569–577
- Substantia nigra pars reticularis (SNr):
 - Parkinson's disease neurochemistry, 481–483
- Sulpiride:
 - Tourette's syndrome therapy, 276
- Superoxide dismutase (SOD):
 - amyotrophic lateral sclerosis and, 225–227
 - stroke management, 371–372
- Supersensitization:
 - melatonin receptors, 54
- Suprachiasmatic nucleus (SCN):
 - animal models, 10–11
 - output pathways, 12–13
 - circadian rhythms and
 - antidepressant effects, 18–20
 - inputs and outputs, 46–47
 - period and phase, 13–15
 - prokineticin regulation, 167–168
 - in cockroach, 10
 - melatonin production, 40–41
 - melatonin receptors in
 - clock gene expression, 50–51
 - desensitization function, 52–53
 - localization, signaling, and function, 47–50
 - regulatory mechanisms, 51–54
 - supersensitization, 54
 - therapeutic targeting of:
 - sleep mechanisms, 57–58
- Surgical treatment:
 - epilepsy, 426
- SVZ compound:
 - neurogenesis and, 209–210
- Sympathetic nervous system (SNS):
 - adipose tissue, 789–797
 - adipocyte proliferative capacity, 797–799
 - anterograde tract-testing, SNS to WAT, 792–797
 - retrograde tracing neuroanatomical studies, 790–792
 - ghrelin effects, 774
 - leptin and energy expenditure, 746
- Synapses:
 - hypocretins at, 130
 - neurotrophins and, 239
- Synaptic vesicle protein SV2A:
 - antiepileptic drug modulation, 414
 - epilepsy efficacy, 417–418
- Synphilin-1* point mutation:
 - familial Parkinson's disease and, 540
- α -Synuclein mutations:
 - familial Parkinsonism and
 - PARK1 and PARK4 mutations, 528–530
 - parkin* gene degradation, 532
 - Parkinson's disease genetics
 - invertebrate models, 570–577
- Tat protein:
 - human immunodeficiency virus, 701–703
 - alcohol abuse and, 716
 - apoptosis and, 705

- excitotoxicity cell death, 705–706
- methamphetamine/cocaine abuse and, 714–715
- nigrostriatal system and, 709
- opioid abuse and, 715–716
- Temperature:
 - circadian rhythms, 5
- Temporal lobectomy:
 - epilepsy, 426
- Temsirolimus (CCI-779):
 - multiple sclerosis therapy, 688
- Teratogenicity:
 - antiepileptic drugs, 424–425
- Δ^9 -Tetrahydrocannabinol:
 - antiobesity therapy, 825–828
 - neuroinflammation and
 - receptor modulation, 641–642
- Tetraspan proteins:
 - structure and function, 607–608
- Therapeutic plasma exchange (TPE):
 - multiple sclerosis acute relapse, treatment of, 673–674
- Thermogenesis:
 - glyceroneogenesis and, 799–801
- THIP agonist:
 - as sedative/hypnotic, 190
- Thyroid function:
 - ghrelin modulation, 775
- Thyrotropin-releasing hormone (TRH):
 - narcolepsy therapy, 105–106
- Tiagabine:
 - epilepsy efficacy, 417–418
 - GABA_A receptor modulation
 - antiepileptic drugs, 412
- Tiapride:
 - Tourette's syndrome therapy, 276
- Tics. *See* Tourette's syndrome
 - Tourette's syndrome, 264–265, 269–273
 - suppressants, 277
- Timeless gene:
 - melatonin receptors, 51
- Time window paradigm:
 - stroke management, 360–361
- TNF-related apoptosis-inducing ligand (TRAIL):
 - inflammation mechanisms, 625
 - proinflammatory pathway modulation, 639
- Toll-like receptors (TLR):
 - inflammation and, 631–632
- Topiramate:
 - as antiepileptic
 - ionotropic glutamate receptor
 - modulation, 413
 - target interactions, 415–416
 - epilepsy efficacy, 417–418
 - for secondary generalized epilepsy, 424
- Torpedo* electroplax:
 - GABA_A receptor structure
 - sedative/hypnotic mechanisms, 178–180
- Tourette's syndrome:
 - clinical course, 266
 - comorbid disorders, 266
 - diagnostic criteria, 263–264
 - environmental factors, 267–269
 - epidemiology, 266
 - future research issues, 278
 - genetics, 266–267
 - pathophysiology, 269–273
 - neuroanatomical abnormalities, 269–270
 - neurochemical abnormalities, 270–273
 - second-messenger systems, 273
 - phenomenology, 264–265
 - treatment, 273–278
 - adrenergic agonists, 276–277
 - antidopaminergic agents, 276
 - neuroleptics, 273–276
 - nonpharmacological treatments, 277–278
 - tic suppressants, 277
- Toxicity effects:
 - antiepileptic drugs, 420–421
- Tranquilizers:
 - structure and function, 187
- Transforming growth factor- α (TGF α):
 - suprachiasmatic nucleus, 13
- Transforming growth factor- β (TGF β):
 - structure and function, 222–223
- Transforming growth factor family:
 - inflammation mechanisms, 640–641
- Transient receptor potential V1 (TRPV1)
 - receptors:
 - pain perception and management, 229
- Transition metals:
 - Parkinson's disease and
 - invertebrate models, 576–577
- Traumatic brain injury (TBI):
 - characteristics and classification, 443–446
 - future research issues, 454
 - neural regeneration, 452–453
 - neuroplasticity, 453–454
 - neuroprotective agents, 446–451
 - anti-inflammatory agents, 447–449

- free-radical scavengers, 447
 - neuroactive steroids/neurosteroids, 450–451
 - neurotransmitter agonists/antagonists, 449–450
- Tricyclic antidepressants:
 - attention-deficit hyperactivity disorder therapy, 299
 - Parkinson's disease and, 501
- Trihexyphenidyl:
 - Parkinson's disease treatment, 496
- TrkA receptors:
 - cognitive function and, 228
 - neurotrophins and, 238
 - pain perception and management, 229
 - TrkAIII splice variants, 245–246
- TrkB receptors:
 - amyotrophic lateral sclerosis and, 226–227
 - neurotrophic factor expression, 222–224
 - obesity and weight control, 229–230
 - Parkinson's disease and, 228–229
 - splice variants, 245–246
 - synaptic function and, 239
- TrkC receptors:
 - neuropathies and, 227
 - splice variants, 245–246
- Tryptophan:
 - inflammation and metabolism of, 644
- Tumor necrosis factor- α (TNF α):
 - human immunodeficiency virus nigrostriatal system and, 710
- Tumor necrosis factor (TNF):
 - neuroinflammation
 - proinflammatory pathway modulation, 638–639
 - p75 neurotrophin receptor structure and, 240–241
- Tyrosine kinase receptors. *See also* specific receptors, e.g. TrkA receptor
 - functional interactions, 247–248
 - neurotrophic factors and, 231–232
 - neurotrophins and, 238
 - signaling mechanisms, 242–246
 - preference determinants, 242
 - protein binding, 243–245
 - signal transduction pathways, 242–243
 - splice variants and, 245–246
 - structure of, 241–242
- Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) mutation:
 - familial Parkinson's disease and, 539–540
- Uncoupling protein:
 - adipose tissue cell types and depots, 786–789
- Upstream techniques:
 - stroke management, 366–373
- Uridine-diphosphoglucose-*N*-acetylglucosamine (UDP-GlcNAc):
 - leptin secretion and, 736
- Vagal nerve stimulation (VNS):
 - epilepsy management, 427
- Valproate:
 - development and testing, 419
 - epilepsy efficacy, 417–418
 - for secondary generalized epilepsy, 424
- Vanilloid receptors:
 - tyrosine kinase receptor signaling, 245
- Vascular cell adhesion molecule-1 (VCAM-1):
 - inflammation mechanisms, 624–625
- Vascular endothelial growth factor (VEGF):
 - prokineticins and, 169–170
- Vascular monoamine transporter (VMAT):
 - narcolepsy therapy
 - amphetamine targeting, 94
- Venlafaxine:
 - attention-deficit hyperactivity disorder therapy, 301
 - autism spectrum disorders, 331
- Ventral tegmental area (VTA):
 - Parkinson's disease neurochemistry and, 481–483
- Ventrolateral preoptic nucleus (VLPO):
 - narcolepsy therapy
 - modafinil, 102–103
- Vesicular monoamine transporter 2 (VMAT2):
 - attention-deficit hyperactivity disorder therapy, 297–298
- Vigabatrin:
 - epilepsy efficacy, 417–418
 - GABA_A receptor modulation, 412
- Vitamin D:
 - neuroinflammation and, 642–643
- Vitamin D receptor agonists:
 - neuroinflammation and, 643
- Vitamin E:
 - neuroinflammation and
 - antioxidant modulation, 637
- Vitamins:
 - idiopathic Parkinson's disease and, 544–545

- VLPO nucleus:
 sedative/hypnotic action sites,
 182–184
- Voltage-activated sodium channels:
 epilepsy and modulation of, 411
- Vpr protein:
 HIV neurotoxicity and, 703
- Vpu protein:
 HIV neurotoxicity, 704
- White adipose tissue (WAT):
 classification, 786–789
 glyceroneogenesis control, lipolysis and
 thermogenesis, 799–801
 molecular features, 787–789
 sympathetic nervous system innervation,
 789–797
 anterograde tract-tracing, 792–797
 retrograde neuroanatomical studies,
 790–792
- Withdrawal:
 antiepileptic drugs, 419–420
- Yellow fluorescent protein (YFP):
 Huntingdon's disease trinucleotide repeats
 invertebrate model, 577–579
- YM-872 antagonist:
 stroke management, 361–362
- Yo-yo-ing dyskinesia:
 Parkinson's disease treatment and, 499
- Zeitgeber* time:
 circadian rhythms and, 20–21
 narcolepsy pathophysiology
 hypocretin/orexin system, 88–89
- Ziprasidone:
 autism spectrum disorders, 327–328
 Tourette's syndrome therapy, 275–276
- Zonisamide:
 T-type calcium channel modulation, 413